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Note

Simple high-performance liquid chromatographic assay for benzylamine oxidation products in cell suspensions

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Benzylamine and benzyl alcohol are useful industrial reagents and the related oxidation products, benzaldehyde and benzoic acid, are important food additives. Benzylamine is also a convenient model substrate for studies of monoamine oxidase function. Several assays are available for each of these compounds [1—3], as well as for the major end product of mammalian metabolism of these compounds, hippuric acid [4], but none has been developed which allows simultaneous assay of these components in cell or tissue extracts. In a recent study of benzylamine metabolism in isolated hepatocytes [5], it was necessary to obtain a comprehensive description of the metabolites at low concentrations in cell incubations under a variety of metabolic conditions. For this purpose, a high-performance liquid chromatographic (HPLC) system was developed which allows resolution of benzaldehyde, benzoate, hippurate, benzylamine and benzyl alcohol. This report describes this simple and sensitive assay and discusses its application to measurement of benzaldehyde, benzoate and hippurate in metabolic studies with cell suspensions.

EXPERIMENTAL

Benzylamine, benzaldehyde, benzoic acid, hippuric acid, collagenase, and HEPES [4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid] were purchased from Sigma (St. Louis, MO, U.S.A.). Benzyl alcohol was from Eastman Organic Chemicals (Rochester, NY, U.S.A.). HPLC-grade methanol was from Baker (Phillipsburg, NJ, U.S.A.). Doubly distilled water was used for chromatography and deionized water was used for media for cell preparation and incubations.

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Isolated hepatocytes were prepared from male white rats (Kng:(SD)Br, King Animal Labs., fed ad libitum) as described previously [6] and incubated at 37°C in rotating round-bottom flasks in Krebs–Henseleit medium supplemented with 12.5 m*M* HEPES [6]. Viability was 95–99% as estimated by exclusion of 0.2% Trypan blue. Reactions were terminated by addition of 0.5 ml 3 *M* perchloric acid per ml incubation mixture, and protein was removed by centrifugation.

HPLC was performed with a Beckman Model 334 gradient chromatograph with an autosampler and a variable-wavelength detector (flow-cell volume, 20 μl).

Separation of the metabolites was obtained on a 5-μm reversed-phase C_{18} column (25 cm × 4.6 mm, Altex, Ultrasphere-ODS) with a gradient formed from solvent A (50 ml methanol, 550 ml 1% acetic acid, 1 ml ethyl acetate, final pH adjusted to 4.2 with 10 *M* potassium hydroxide) and Solvent B (420 ml methanol, 180 ml 1% acetic acid, 1 ml ethyl acetate, final pH adjusted to 4.2 with 10 *M* potassium hydroxide). The program was as follows: 6 min at 100% solvent A, 10-min linear gradient from 100% solvent A to 100% solvent B, hold 4 min at 100% solvent B, 1-min linear gradient from 100% solvent B to 100% solvent A, hold 10 min at 100% solvent A before injecting another sample. Flow-rate was maintained at 1 ml/min and chromatograms were run at room temperature. Injection volumes were routinely 100 or 200 μl; comparison of recoveries of the same amount of standard injected in different volumes (5–200 μl) showed that recovery was independent of injection volume over this range. Quantitation was performed from the absorbance at 250 nm by both peak height measurements and with a Hewlett-Packard Model 3390A integrator. Integration values are presented, but were proportionate to peak height down to the limits of the assay as expressed below.

RESULTS AND DISCUSSION

Because most cellular metabolites are more polar than benzylamine and its metabolites, an inital isocratic period with 100% solvent A allowed rapid elution of these compounds with retention of the compounds of interest. Separation was highly pH-dependent over the range of 3.3 to 6.0, and optimal separation was obtained at 4.2 with conditions as described in Fig. 1. Separation was very reproducible over a period of several months.

Studies of the recoveries of benzaldehyde, benzoic acid and hippuric acid from cell incubations (Table I) show good recovery can be obtained by precipitation of protein with acid and directly analyzing the protein-free supernatant following centrifugation. For critical analytical studies, correction for incomplete recovery would be necessary, especially if cell content is not constant for different assays. Some retention of the compounds on the columns occurred, but this was not a problem for routine measurements except for initial runs following prolonged washes with methanol or following analysis of relatively high quantities (> 50 nmol). Carry-over from consecutive runs was typically less than 5%, but was sufficient to require a blank run prior to assay of zero time or control incubations. Standard error for integrations from four successive 20-μl injections was less than ± 5%.

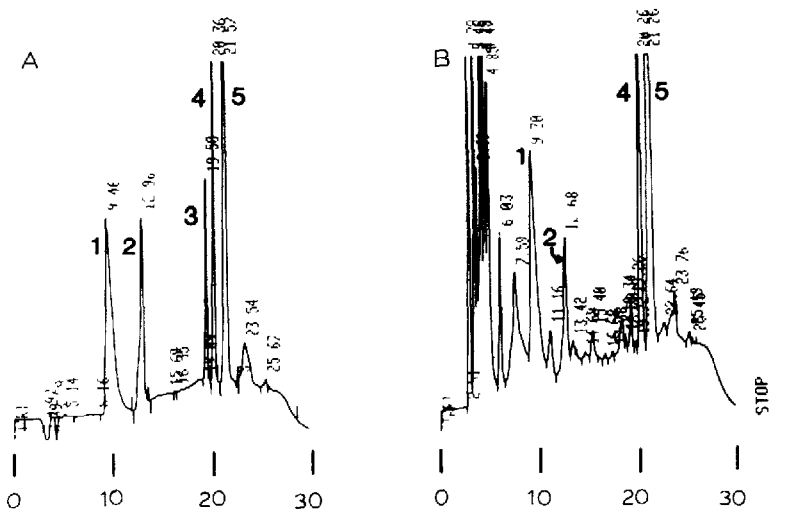

Fig. 1. Separation of benzylamine (1), hippuric acid (2), benzyl alcohol (3), benzoic acid (4) and benzaldehyde (5) by HPLC (A) 100 μl of a standard mixture of 5 m*M* benzylamine, 0.25 m*M* hippuric acid, 1 m*M* benzyl alcohol, 0.5 m*M* benzoic acid and 0.12 m*M* benzaldehyde were injected on a 5-μm C_{18} column pre-equilibrated with 100% solvent A and run as described in the Experimental section. (B) The metabolic products formed by hepatocytes ($2 \cdot 10^6$ cells per ml) from 5 m*M* benzylamine are separated. Cells were incubated at 37°C for 30 min, and the protein was removed by addition of 3 *M* perchloric acid (0.5 ml per ml incubation) and centrifugation. Injection volume was 200 μl. Retention times for standards were: benzylamine, 9.5 min; hippuric acid, 13.0 min; benzyl alcohol, 19.5 min; benzoic acid, 20.4 min; benzaldehyde, 21.6 min.

TABLE I

RECOVERY OF EXOGENOUS METABOLITES ADDED TO HEPATOCYTE SUSPENSIONS

Metabolites were added at either 2.25 μmol per 10^6 cells or 90 nmol per 10^6 cells; 1-ml aliquots were immediately treated with 0.5 ml of 3 *M* perchloric acid per ml sample and protein was removed by centrifugation. Injection volume for assay was 20 μl. Percent recovery from cells was calculated as the amount recovered from cell suspensions relative to the amount recovered under identical conditions without cells. Values are mean ± standard error for four assays.

Metabolite	Added (nmol)	Recovered —cells (nmol)	Recovered +cells (nmol)	Recovery from cells (%)
Benzaldehyde	30	25.5 ± 1.8	20.9 ± 1.0	82
	1.2	1.19 ± 0.12	1.01 ± 0.03	86
Benzoate	30	29.7 ± 1.5	24.0 ± 1.3	93
	1.2	0.82 ± 0.10	0.86 ± 0.09	105
Hippurate	30	27.2 ± 0.7	24.6 ± 1.3	91
	1.2	1.13 ± 0.04	1.18 ± 0.14	105

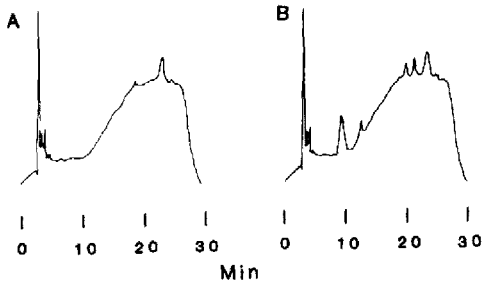

Fig. 2. Illustration of sensitivity of detection of benzylamine oxidation products in isolated hepatocytes. (A) 20 μl of the supernatant from acid-treated hepatocytes (0.5 ml 3 *M* perchloric acid plus 1.0 ml of 10^6 cells per ml) were injected and run as in Fig. 1, except with four-fold higher sensitivity. (B) 20μl of an identical supernatant with added standards, 36 nmol benzylamine, 0.9 nmol hippuric acid, 0 3 nmol benzoic acid and 0.09 nmol benzaldehyde, were run as in (A).

For standards diluted in 1 *M* perchloric acid, quantitation of benzaldehyde was linear over the range of 10 pmol to at least 5 nmol and of benzoate was linear over the range of 50 pmol to at least 25 nmol. Quantitation of hippurate was only linear from 0.25 to 25 nmol because peak broadening occurred with lower amounts. Quantitation of standards added to hepatocytes had a similar sensitivity (see Fig. 2), but in our studies of benzylamine metabolism in hepatocytes [5] only zero-time incubations have concentrations at the lower end of the linear range. In principal, benzylamine and benzyl alcohol could also be quantitated by this approach, but neither assay was found to be practical for cell incubations. The extinction coefficient for benzylamine at 250 nm is relatively low and because of peak broadening and benzylamine retention by the column, acceptable quantitation was not achieved. Benzyl alcohol is not produced from benzylamine at a level detectable in liver cell incubations and consequently, assay of this metabolite has not been pursued.

Benzylamine is metabolized in tissues by monoamine oxidase and is readily assayed in purified preparations of the enzyme by measuring the increased absorbance at 250 nm due to benzaldehyde formation [3]. However, in cells, benzaldehyde is rapidly oxidized to benzoic acid, principally catalyzed by an NAD^+-dependent cytosolic enzyme [7, 8]. Benzoate is further metabolized in mitochondria by conjugation with glycine to form hippurate [9]. Since monoamine oxidase has a rather high K_m value for benzylamine, assay of the mixture of products is necessary for studies with cells and tissues. The current method provides a simple and direct assay for this purpose. The method has been suitable for measuring metabolism of benzylamine, benzaldehyde and benzoate in mitochondria and other subcellular fractions as well as in intact cell suspensions. Acid extracts of tissue samples were stable at $-20°C$ for at least two weeks. The assay is sensitive enough for short incubations (i.e., 3—5 min) and also for systems with low cell concentrations. The entire assay time for the method as described was 31 min between injections. This program can be shortened to 21 min if resolution of benzylamine and benzyl alcohol are not needed.

In conclusion, an HPLC method has been developed which provides a simple

and sensitive assay for oxidation products of benzylamine in cell suspensions and tissues. Suitable quantitation of benzaldehyde, benzoate and hippurate can be obtained in a single run. Direct detection by UV absorption allows accurate quantitation at concentrations of interest in biological tissues.

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