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DETERMINATION OF ANTHRANILATE, 3-HYDROXYANTHRANILATE AND 2,3-DIHYDROXYBENZOATE IN THE ENZYME ASSAY OF ANTHRANILATE HYDROXYLASE BY THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatography technique using UV detection is reported for the assay of anthranilate 2,3-dioxygenase (deaminating) activity. The substrate anthranilate, the main product 2,3-dihydroxybenzoate and the by-product 3-hydroxyanthranilate are determined quantitatively in the presence of biological material. After addition of benzoic acid as internal standard, the substances of interest are separated isocratically on a reversed-phase C_{18} column with a detection sensitivity of 0.5 nmol and retention times of 3.5–10 min. A thin-layer chromatographic and fluorimetric method is also described.

INTRODUCTION

Nicotinamide nucleotides are synthesized in microorganisms under aerobic conditions via anthranilate (AA), tryptophan, kynurenine and 3-hydroxyanthranilate (3HOAA). A "short-cut" pathway has been postulated in which AA is directly hydroxylated to 3HOAA, eliminating eight enzymatic steps[1].

We have found that the enzyme which directly hydroxylates AA to 3HOAA is anthranilate hydroxylase (E.C.1.14.12.2; listed as anthranilate 2,3-dioxygenase, deaminating), which mainly catalyses the formation of 2,3-dihydroxybenzoate (2,3DHB) from AA[2]. Further studies of this enzyme require an enzymatic test by which not only substrate AA and main product 2,3DHB, but also traces of the additional product 3HOAA, can be determined quantitatively.

The separation and quantitation of various metabolites of the tryptophan–kynurenine pathway have been reported in numerous publications, using such methods as paper chromatography[3], thin-layer chromatography[4–10], ion-exchange chromatography[10–12], adsorption chromatography[13], molecular sieve chromatography[14], gas chromatography[15,16] and high-performance liquid chromatography (HPLC)[17–20]. To date, however, no single technique has been reported by which all three metabolites, AA, 3HOAA and 2,3DHB, could be detected.

This paper describes a method for the simultaneous determination of AA, 2,3DHB and 3HOAA in the reaction mixture of the anthranilate hydroxylase assay.

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EXPERIMENTAL

Instrumentation

HPLC separation was performed with a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Model U6K injection valve with a 2-ml sample loop (Waters) and a LiChrosorb C_{18} reversed-phase column (250 × 4.6 mm I.D., average particle size 10 μm; Knauer, Berlin, G.F.R.). The eluate was monitored with a variable-wavelength UV detector (Model LC3, Pye Unicam, Cambridge, Great Britain). Peak areas were calculated with an integrator (Model Minigrator DP 88; Spectra-Physics, Darmstadt, G.F.R.). In addition, all chromatograms were recorded on a 10-mV Servogor recorder (Model 210; BBC Metrawatt, Nürnberg, G.F.R.) at a chart speed of 5 mm/min. Fluorimetric determinations were carried out with a Perkin-Elmer spectrophotofluorimeter (Model MPF 2A; Perkin-Elmer, Norwalk, CT, U.S.A.), coupled to a 10-mV recorder.

Materials

All solvents and standards were of analytical-reagent grade. Methanol, benzene, dioxane, mercaptoethanol, orthophosphoric acid (85%), anthranilic acid and benzoic acid were purchased from Merck (Darmstadt, G.F.R.). 2,3-dihydroxybenzoic acid were obtained from ICN, K&K Labs. (Plainview, NY, U.S.A.), catechol and Avicel-cellulose SF from Serva (Heidelberg, G.F.R.) and glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH from Boehringer (Mannheim, G.F.R.).

Procedures

Enzyme preparation. Aspergillus niger, strain DSM 734, was grown at 30°C on a minimal medium according to Byrde *et al.*[21] with tryptophan (1 g/l). The mycelium was harvested after about 48 h and washed with distilled water. After grinding in a special mill[22], the homogenate was centrifuged at 20,000 *g* and at 100,000 *g*. Anthranilate hydroxylase was determined in the supernatant crude extract.

Assay of anthranilate hydroxylase activity. Activity of anthranilate hydroxylase was assayed according to Langenbach-Schmidt[23]. The incubation mixture of 1 ml contained 0.8 μmol of AA, 0.4 μmol of NADPH, 0.4 μmol glucose-6-phosphate, 150 mU of glucose-6-phosphate dehydrogenase, 17 μmol of potassium phosphate buffer (pH 8.0) and 0.5 ml of crude extract. The reaction was initiated by addition of enzyme, incubated at 30°C and stopped after 15 min by addition of 0.1 ml of 0.35 *M* sulphuric acid. When anthranilate hydroxylase activity was determined by HPLC, 0.1 ml of methanol containing the internal standard (benzoic acid) was added.

AA, 3HOAA, 2,3DHB, benzoic acid and catechol, a catabolite of 2,3-DHB, were extracted twice with 2 ml of diethyl ether. The ether layers were combined and evaporated to dryness in a water-bath at 40°C and the residue was dissolved in 0.2 ml of methanol. Aliquots of this methanolic extract were analysed by thin-layer chromatography (100 μl) or HPLC (25 μl).

RESULTS AND DISCUSSION

Separation of AA, 2,3DHB and 3HOAA by thin-layer chromatography

A 100-μl volume of methanol, containing AA, 3HOAA and 2,3DHB, were applied in a band 3 cm long to a glass plate (20 × 20 cm) coated with 0.25 mm Avicel-cellulose SF powder. The plate was developed with benzene–methanol (50:10) at room temperature for 1 h in the dark. The spots were located under short- and long-wavelength UV light. Because of the very different R_F values (AA 0.86; 3HOAA 0.41; 2,3DHB 0.20), small amounts of 3HOAA (0.5 nmol) can be detected as well as larger amounts of AA (400 nmol) and 2.3DHB (100 nmol) (Fig. 1).

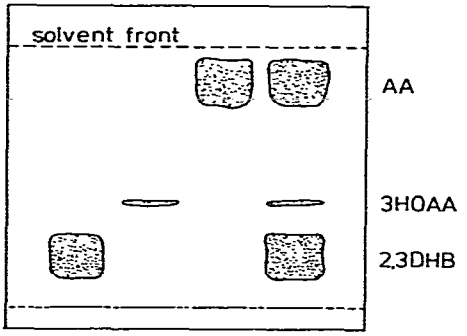

Fig. 1. Thin-layer chromatogram of AA (400 nmol), 2,3DHB (100 nmol), 3HOAA (0,5 nmol) and a mixture of these substances. Adsorbent, Avicel-cellulose SF, 0.25 mm; solvent, benzene–methanol (50:10).

For quantitative determination, the 3HOAA or AA spots were scraped off into test-tubes and shaken with 0.5 ml of methanol (stabilized with 1% of mercaptoethanol) for 30 min. Dioxane (4 ml) was then added[24]. After centrifugation, the fluorescence of the supernatant was measured at the excitation and emission maxima of 3HOAA (345 and 410 nm) or AA (342 and 395 nm).

The fluorescence intensity of both substances varies linearly with concentration in the range of 1–10 nmol per 4.5 ml of methanol–dioxane–mercaptoethanol mixture. The within-day precision of quantitative results gave a relative standard deviation of *ca.* 4.4% (n = 14) and the average recoveries were 81% for both 3HOAA and AA. 2,3DHB can be determined only qualitatively, because the fluorescence intensity does not vary linearly with concentration.

AA, 3HOAA and 2,3DHB can also be separated well by thin-layer chromatography in a phenol–water system (160 g + 40 ml). Under these conditions, however, the fluorescence is so reduced through quenching that a quantitative determination of 3HOAA and AA is impossible.

Determination by HPLC

3HOAA, catechol, AA and 2,3DHB are separated on a reversed-phase C_{18} column by injection of 25 μl of a methanolic solution and detected at 213 nm, where traces of all substances, especially of 3HOAA, can be identified (Fig. 2). The separation is carried out isocratically at room temperature at a flow-rate of 1.8 ml/min

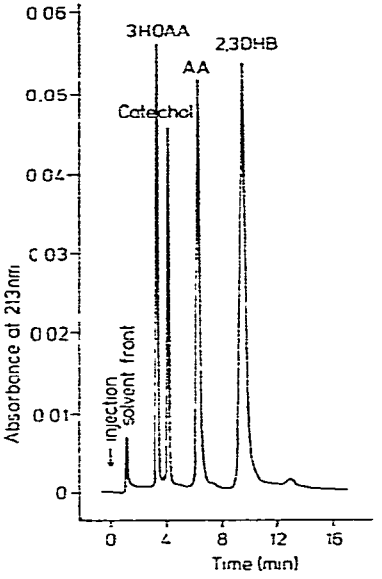

Fig. 2. High-performance liquid chromatogram of catechol (2.5 nmol), 3HOAA (1.25 nmol), AA (2.5 nmol) and 2,3DHB (2.5 nmol). Column, reversed-phase LiChrosorb C_{18} (250 × 4.6 mm I.D.); eluent, methanol–water–85% orthophosphoric acid (30:70:0.3); flow-rate, 1.8 ml/min; wavelength, 213 nm.

within 20 min. The mobile phase used is 300 ml of methanol plus 3 ml orthophosphoric acid diluted to 1000 ml with doubly distilled water. Before use, the solution is filtered through a Millipore FHUP 04700 filter (0.5 μm) and degassed ultrasonically. 3HOAA, catechol, AA and 2,3DHB have capacity ratios (k') (based on unretained solvent peak) of 1.42, 1.9, 3.4 and 5.6, respectively. The high resolutions of 2.4, 4.7 and 4.5 for 3HOAA–catechol, catechol–AA and AA–2,3DHB, respectively, are necessary for separating unknown impurities in the crude extract, occurring in the enzyme assay. The separation becomes worse with higher concentrations of methanol in the mobile phase by decreasing the retention times of 2,3DHB and benzoic acid, and also with small amounts of orthophosphoric acid by increasing the retention times of AA and 3HOAA.

The calibration graphs (Fig. 3) show constant relationships between peak area and concentration from 0.5 nmol to at least 20 nmol of 3HOAA or 40 nmol of 2,3DHB and AA or 300 nmol of benzoic acid. Standard curves were fitted by regression analysis and the correlation coefficients were >0.998 between theoretical concentrations and peak areas for all components. Also, the correlation coefficients between concentration and the peak-area ratio of the substance being analysed to the internal standard were >0.999.

The assay demonstrated a recovery of about 99% relative to benzoic acid as the internal standard and average relative standard deviations of 0.7% and 3.0% for within-day and between-day runs, respectively (Table I).

The minimal detectable concentrations in the absence of biological material from the enzyme preparation are 0.02 nmol of 3HOAA, 0.07 nmol of AA and 0.12 nmol of 2,3DHB.

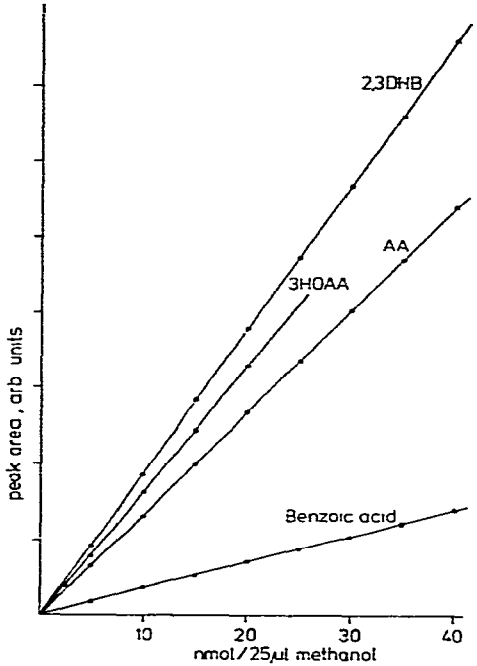

Fig. 3. Calibration graphs of peak area *versus* amount of AA, 2,3DHB, 3HOAA, catechol and benzoic acid injected.

TABLE I

PRECISION OF HPLC ASSAY

The injection volume was 25 µl. The relative standard deviation was calculated using benzoic acid as the internal standard.

Compound	*No. of samples*	*Theoretical concentration (nmol per 25 µl)*	*Relative standard deviation (%)*	
			Within-day	*Between-day*
3HOAA	6	0.25	0.6	1.0
	5	2.0	0.5	1.0
	6	5	0.7	1.3
2,3DHB	4	6	1.6	2.3
	4	18	0.5	2.3
	4	30	0.3	2.4
AA	4	6	1.4	5.2
	4	18	0.5	5.7
	4	30	0.3	5.4

For determination of anthranilate hydroxylase activity, the enzyme assay is extracted with diethyl ether and dissolved in methanol as described above. A 25-μl volume of the methanolic phase is injected into the column (Fig. 4). Benzoic acid is used as an internal standard because it is extracted by diethyl ether and dissolves in methanol just as AA, 3HOAA and 2,3DHB. In addition, it can be separated well from the other substances on the reversed-phase column (Fig. 4). A 1600-nmol amount of benzoic acid, dissolved in 0.1 ml of methanol, is added after stopping the enzyme reaction with sulphuric acid.

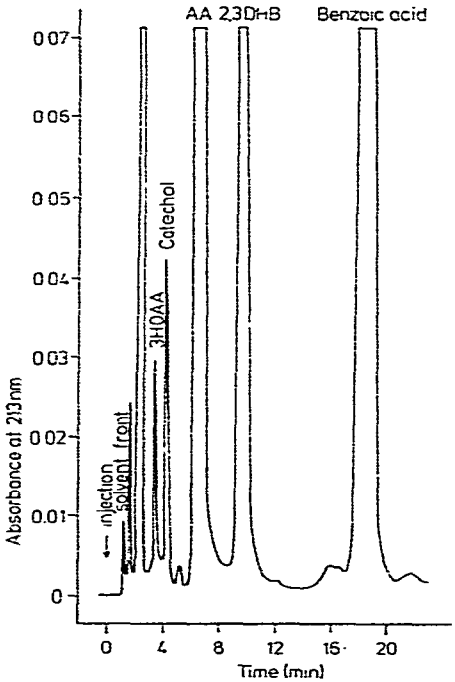

Fig. 4. High-performance liquid chromatogram of the methanolic extract of the anthranilate hydroxylase assay. Column, reversed-phase LiChrosorb C_{18} (250 × 4.6 mm I.D.); internal standard, benzoic acid; eluent, methanol–water–85% orthophosphoric acid (30:70:0.3); flow-rate, 1.8 ml/min; wavelength, 213 nm.

Using a μBondapak C_{18} column (particle size 10 μm, 300 × 3.9 mm I.D.; Waters) instead of the LiChrosorb C_{18} column, the quantitation of 3HOAA cannot be achieved under the above mentioned conditions because it cannot be separated from an unknown metabolite of the enzyme reaction (Fig. 4). Also, catechol, a catabolite of 2,3DHB, elutes together with AA on the former column.

The practical advantages of HPLC over thin-layer chromatography are rapidity, good reproducibility and high sensitivity.

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REFERENCES

1 R. Bode, F. Böttcher, D. Birnbaum and I. A. Samsonova, *Z. Allg. Mikrobiol.*, 15 (1975) 149–155.
2 H.-H. Schott, K. Zinke and D. Langenbach-Schmidt, in preparation.
3 D. Coppini, C. A. Benassi and M. Montorsi, *Clin. Chem.*, 5 (1959) 391–401.
4 C. A. Benassi, F. M. Veronese and E. Gini, *J. Chromatogr.*, 14 (1964) 517–519.
5 M. P. Walsh, *Clin. Chim. Acta*, 11 (1965) 263–267.
6 I. Smith, in I. Smith (Editor), *Chromatographic and Electrophoretic Techniques*, Heinemann, London, 1969, pp. 104–169.
7 J. B. Weiss and M. Spencer, in I. Smith (Editor), *Chromatographie and Electrophoretic Techniques*, Heinemann, London, 1969, pp. 793–799.
8 R. Humbel and C. Marsault, *J. Chromatogr.*, 79 (1973) 347–348.
9 P. Calandra, *Acta Vitamin. Enzymol.*, 28 (1974) 189–194.
10 K. M. Morris and R. J. Moon, *Anal. Biochem.*, 61 (1974) 313–327.
11 S. L. Trompsett, *Anal. Chim. Acta*, 24 (1961) 438–443.
12 A. F. Heeley, *Clin. Sci.*, 29 (1965) 465–473.
13 M. Y. Kamel and R. R. Hamed, *J. Chromatogr.*, 157 (1978) 440–444.
14 E. Stratakis, *Insect Biochem.*, 6 (1976) 149–151.
15 T. Noguchi, H. Kaseda, N. Konishi and R. Kido, *J. Chromatogr.*, 55 (1971) 291–295.
16 K. Hirano, K. Mori, N. Tsuboi, S. Kawai and T. Ohno, *Chem. Pharm. Bull.*, 20 (1972) 1412–1416.
17 M. Ghebregzabher, S. Rufini, M. G. Castellucci and M. Lato, *J. Chromatogr.*, 222 (1981) 191–201.
18 E. Grushka, E. J. Kikta, Jr. and E. W. Naylor, *J. Chromatogr.*, 143 (1977) 51–56.
19 A. M. Krstulovic and C. Matzura, *J. Chromatogr.*, 163 (1979) 72–76.
20 S. Yong and S. Lau, *J. Chromatogr.*, 175 (1979) 343–346.
21 R. J. W. Byrde, J. F. Harris and D. Woodcock, *Biochem. J.*, 64 (1956) 154–160.
22 H. Weiss, G. v. Jagow, M. Klingenberg and T. Bücher, *Eur. J. Biochem.*, 14 (1970) 75–82.
23 D. Langenbach-Schmidt, *Dissertation*, Justus Liebig-Universität Giessen, Giessen, 1979.
24 M. Watanabe and K. Hayashi, *Clin. Chim. Acta*, 37 (1972) 417–422.