CLINICAL ANALYSIS OF AROMATIC AMINO ACIDS IN SERUM BY REVERSED-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

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The assay of free serum tyrosine (Tyr) and phenylalanine (Phe) is of clinical relevance both for the detection of hypertyrosinaemia and phenylketonuria (PKU) in neonates, and for monitoring response to long-term dietary treatment. While the Guthrie test (1961) is of proven value for screening neonates for PKU, a rapid assay for serum Phe is required to give diagnostic feedback to the clinician. Conventional ion-exchange chromatography or spectrofluorimetric methods (McCaman and Robins 1962) are lengthy, whereas reported methods by high pressure liquid chromatography (HPLC) require chemical derivatisation or gradient elution facilities, and have not been quantitatively evaluated for PKU (Lindroth & Mopper 1979; Neckers et al 1981). The present work describes rapid, sensitive procedures for the micro-assay of serum Phe and Tyr in clinical studies on in-born errors of metabolism.

The aromatic amino acids in deproteinised samples were well resolved on 5 μM ODS-Hypersil in a 250 x 5 mm column, the eluent being methanol/KH_2PO_4 (50 mM) at pH 4.00 (13: 87% v/v). The phase capacity ratios (k') were: Tyr 0.57, Phe 1.84 tryptophan 3.80. As an internal standard (IS) β-2-thienylalanine (used as an inhibitor in the Guthrie test) was satisfactory (k', 0.87). Rapid analysis of Phe at the optimum flow rate (1.2 ml min^{-1}) was achieved by sequencing sample injections (3 μl) at 10 minute intervals. No interference was observed by known aromatic amino acid metabolites. Only two of six deproteinisation procedures examined were suitable. The simpler method, using acetonitrile at twice sample volume, yielded a sharp peak unresolved from Phe. Other deproteinisation procedures yielded broad interfering reagent peaks. Zinc hydroxide, however, yielded a clear supernatant and no interference. The reagent is generated in situ by adding excess 10% $ZnSO_4$ (100 μl) and 0.5M NaOH (50 μl) to 50 μl serum, followed by gentle shaking on a spiral mixer (2 min) and centrifugation at 120 g (5 min).

Column performance (N, theoretical plates m^{-1}) and k' were taken as parallel optimisation criteria for each eluent parameter in turn. Under the conditions described, optimum resolution was observed for Tyr, IS, Phe, Trp, and polar components at the solvent front (N > 40,000 plates m^{-1}). Column life was extended by use of an integral 'guard' column packed with 5 μM ODS-Hypersil. High sensitivity was obtained by UV-detection at 210 nm, the detection limits (twice baseline noise) being: Phe, 1.3 ng (7.9 pM); Tyr, 1.0 ng (5.5 pM). Calibration curves of peak height response ratios for Phe:IS and Tyr:IS in spiked horse serum containing IS at 1000 μM were linear over the clinical ranges of interest and regressed through the origin. The 95% confidence limits (n = 12) were: Phe 1000 ± 32 μM; Tyr, 200 ± 44 μM. Recovery of 1000 μM Phe added to serum was 100.1% (RSD, 0.98%; n = 5) and for Tyr (250 μM) it was 103.8% (RSD = 1.48%; n = 5). The HPLC procedure was compared with an automated version of the McCaman and Robins method (developed at the RHSC, Edinburgh) by assaying duplicate serum standards spiked with Phe from 100-1300 μM, when the correlation coefficient was 0.9994 (p = 0.95; n = 12).

This flexible and rapid HPLC procedure has been applied to the assay of paediatric PKU samples for monitoring serum Phe response to dietary treatment. The simultaneous assay of Phe and Tyr offers a sensitive method for diagnosis of incipient Tyr deficiency in PKU, and for measuring Phe: Tyr ratios in loading tests for the detection of heterozygote parents in genetic counselling.

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