

Technical methods

A two-dimensional chromato-bioautographic method for complete separation of individual plasma cobalamins

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In concentrated aqueous solution methylcobalamin, cyanocobalamin, deoxyadenosylcobalamin, and hydroxocobalamin are readily separable by unidirectional chromatography on paper or thin layers of cellulose (Suomela, 1967). No location reagent is required since the cobalamins are visible as red or orange spots.

The total cobalamin concentration of a plasma extract is between 10^{-5} and 10^{-7} that of an aqueous solution used for visible loading of chromatograms. These small amounts lie far below the detection limit of normal chemical methods and necessitate the use of bioautography for location. Lindstrand and Ståhlberg (1963) were the first to carry out paper chromatography and bioautography of plasma cobalamins

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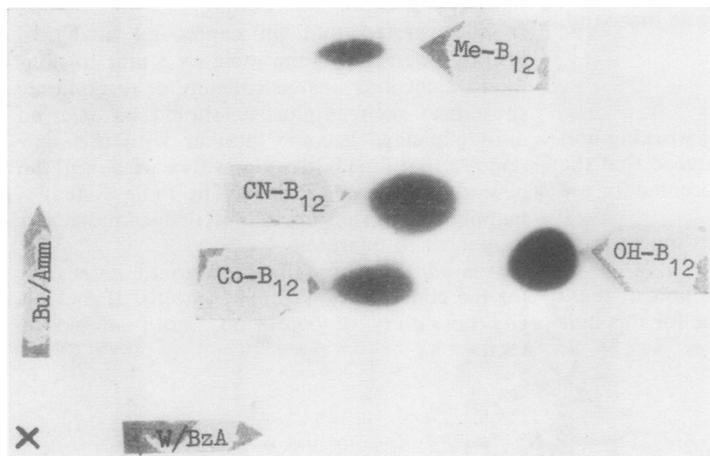


Fig. 1 Separation and location of cobalamin markers in aqueous solution by two-dimensional chromatography and bioautography. 5-Deoxyadenosylcobalamin is labelled as 'Co-B₁₂'.

and more recently a method has been described (Linnell, Mackenzie, and Matthews, 1969b; Linnell, Mackenzie, Wilson, and Matthews, 1969c) which uses thin-layer chromatography and is applicable to small quantities (2 ml) of plasma. Neither of these methods will achieve clear separation of the four cobalamins apparently existing in human plasma (methylcobalamin, cyanocobalamin, deoxyadenosylcobalamin, and hydroxocobalamin). Though our method gives good separation of methylcobalamin, cyanocobalamin, and deoxyadenosylcobalamin plus hydroxocobalamin, the latter two compounds do not separate adequately at the low concentrations present in plasma.

A method has now been developed giving complete separation of these four compounds.

Method

The method of extracting and concentrating plasma cobalamins is as previously described (Linnell *et al.*, 1969c). Of the plasma extract, 4 μ l, is applied to a point 2.5 cm from the corner of a 20 cm square plate previously coated with a 3:1 mixture of Whatman CC 41 cellulose and Merck silica gel G. One μ l of an aqueous solution containing 50 pg each of the four cobalamins per μ l is applied in like manner to a second plate. Cobalamins are separated by ascending chromatography in sec. butanol/amm. water (75:2:25) for three hr at 20-23°C. The plates are then removed from this solvent, dried, and re-developed at right angles to the direction of the first separation for 30 min in water saturated with benzyl alcohol at 20-23°. Up to this point, all steps are carried out in darkness or by red photographic 'safelight' in order to prevent the photolytic conversion of other cobalamins to hydroxocobalamin. The plates are finally dried in daylight. Bioautograms are prepared using B₁₂-deficient agar medium inoculated with a B₁₂-requiring mutant of *E. coli* and containing a tetrazolium indicator (Linnell *et al.*, 1969c).

Results

Figure 1 shows the separation of aqueous cobalamin standards (50 pg of each) by the system described. The first solvent separates methylcobalamin (R_f 0.35) from cyanocobalamin (R_f 0.20) and from 5-deoxyadenosylcobalamin plus hydroxocobalamin (R_f 0.13). In the second solvent, a clear separation is achieved between hydroxocobalamin (R_f 0.85) and 5-deoxyadenosylcobalamin (R_f 0.60).

Figure 2 shows a bioautogram obtained from the plasma of a normal healthy subject. An estimate of each cobalamin obtained by densito-

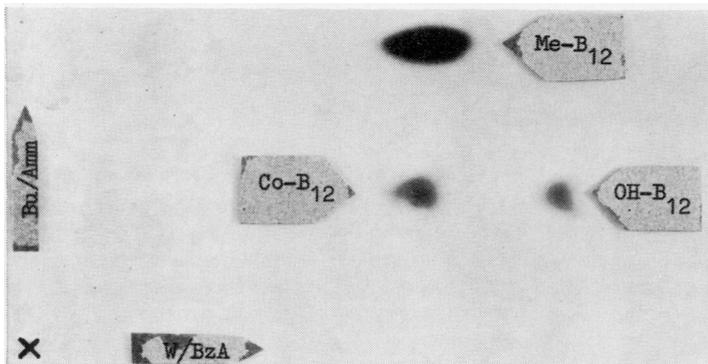


Fig. 2 Separation and location of cobalamins in extract of plasma from healthy subject. Cyanocobalamin, which is never present in more than trace amounts in normal plasma, and often undetectable, did not appear in this sample.

metric scanning and comparison with standards similarly run (Linnell, Hoffbrand, Peters, and Matthews, 1971) gave the following result: methylcobalamin 418 pg/ml; cyanocobalamin none detected; deoxyadenosylcobalamin 163 pg/ml; hydroxocobalamin 129 pg/ml. The result in a further normal subject was as follows: methylcobalamin 307 pg/ml; cyanocobalamin ≤ 5 pg/ml; deoxyadenosylcobalamin 162 pg/ml; hydroxocobalamin 51 pg/ml.

Comment

The ability to achieve complete separation of small amounts of the various cobalamins is of obvious utility in the study of B_{12} metabolism. Qualitative and quantitative results obtained by one-dimensional chromatography and bioautography of plasma cobalamins in patients with B_{12} deficiency, known derangements of B_{12}

metabolism, and various amblyopias have already been reported (Linnell, Hoffbrand, Peters, and Matthews, 1969a; Linnell, Wilson, and Matthews, 1969d). The technique might be expected to be particularly useful in the study of inborn errors of metabolism involving the coenzyme forms of B_{12} (Morrow, Barness, Cardinale, Abeles, and Flaks, 1969; Levy, Mudd, Schulman, Dreyfus, and Abeles, 1970).

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