CHROM. 4228

Some remarks on the paper chromatographic study of urinary β-aminoisobutyric acid

Besides high and low excretion in normal individuals[1–4], β-aminoisobutyric acid (BAIB) excretion is of interest in a number of clinical conditions[5–9] and is regarded as an index of DNA catabolism in clinical medicine. From the point of view of a clinical biochemist, BAWDEN[10] has critically evaluated the different methods available for the determination of urinary BAIB and has finally advocated ion-exchange desalting followed by uni-dimensional separation in the solvent, *n*-butanol–acetic acid–water (12:3:5). This investigator has laid stress on the removal of urea prior to chromatography, since it interferes with the resolution. In the present paper a solvent which avoids the effect of urea in a similar chromatographic separation is described. Further the failure of the uni-dimensional technique to give unambiguous results, in our hands, is discussed, the impressions being based on a study of urinary excretion of BAIB in 87 individuals. Finally an unpublished paper chromatographic method is referred to in which BAIB is discretely separated from untreated urinary samples. This is of special interest in the light of the fact that the desalting procedures often result in losses of BAIB.

Experimental

Separations were done on 11 × 9 in. strips of filter paper (Whatman No. 1, Chromatographic grade). For uni-dimensional study, a line was drawn 2 in. from the smaller edge. The following spots were applied on the line about 1.5 in. apart

Spot A: acetone–HCl extract[11] equivalent to 30 μg of urine.

Spot B: same as spot A.

Spot C: same as spot A + 0.02 ml of standard BAIB solution (20.6 mg in 80 ml of water containing 20 % isopropanol).

Spot D: 0.02 ml of standard BAIB solution alone.

The paper was rolled into a cylinder and was submitted to an 18-h ascending run in the solvent[12] isopropanol–*n*-butanol–isoamyl alcohol–formic acid–water (5:3:2:1.5:2).

Papers were stained with a 0.2 % solution of ninhydrin in acetone. Solution was made 2 % with respect to pyridine just before staining. Papers were allowed to remain at room temperature for full color development. At this stage, the portion of the chromatogram corresponding to the spot A was cut out to serve as unheated control, while the rest of the paper was heated at 70° for 10 min. Comparison of the amino acids separating from spots A, B, C and D allowed qualitative evaluation of BAIB. In principle the method is similar to that of BAWDEN[10] with the difference that no special effort has been made to remove urea from the samples as this was unnecessary with this solvent system.

Two-dimensional separations were done using the solvent pyridine–*n*-butanol–water (10:10:10)[11] for the first run and the solvent used for the uni-dimensional separation for the second run. An extract equivalent to 45 μg of urine was used in these separations.

Results and discussion

Separation of a mixture of tyrosine, β-aminoisobutyric acid, phenylalanine and urea, in the solvents *n*-butanol–acetic acid–water (5:3:2) and isopropanol–*n*-butanol–isoamyl alcohol–formic acid–water (5:3:2:1.5:2) is shown in Fig. 1. In the first solvent β-aminoisobutyric acid and urea overlap each other (R_F values 0.49 and 0.50, respectively), interfering with the discrete separation and staining of β-aminoisobutyric acid. In the presence of moderate or small amounts of urea, β-aminoisobutyric acid does stain with ninhydrin, although it does not separate as a compact spot. If urea is present in large amounts, even the staining is poor (see Fig. 1). In the case of the second solvent, β-aminoisobutyric acid is seen as a discrete spot on the top of a large urea spot

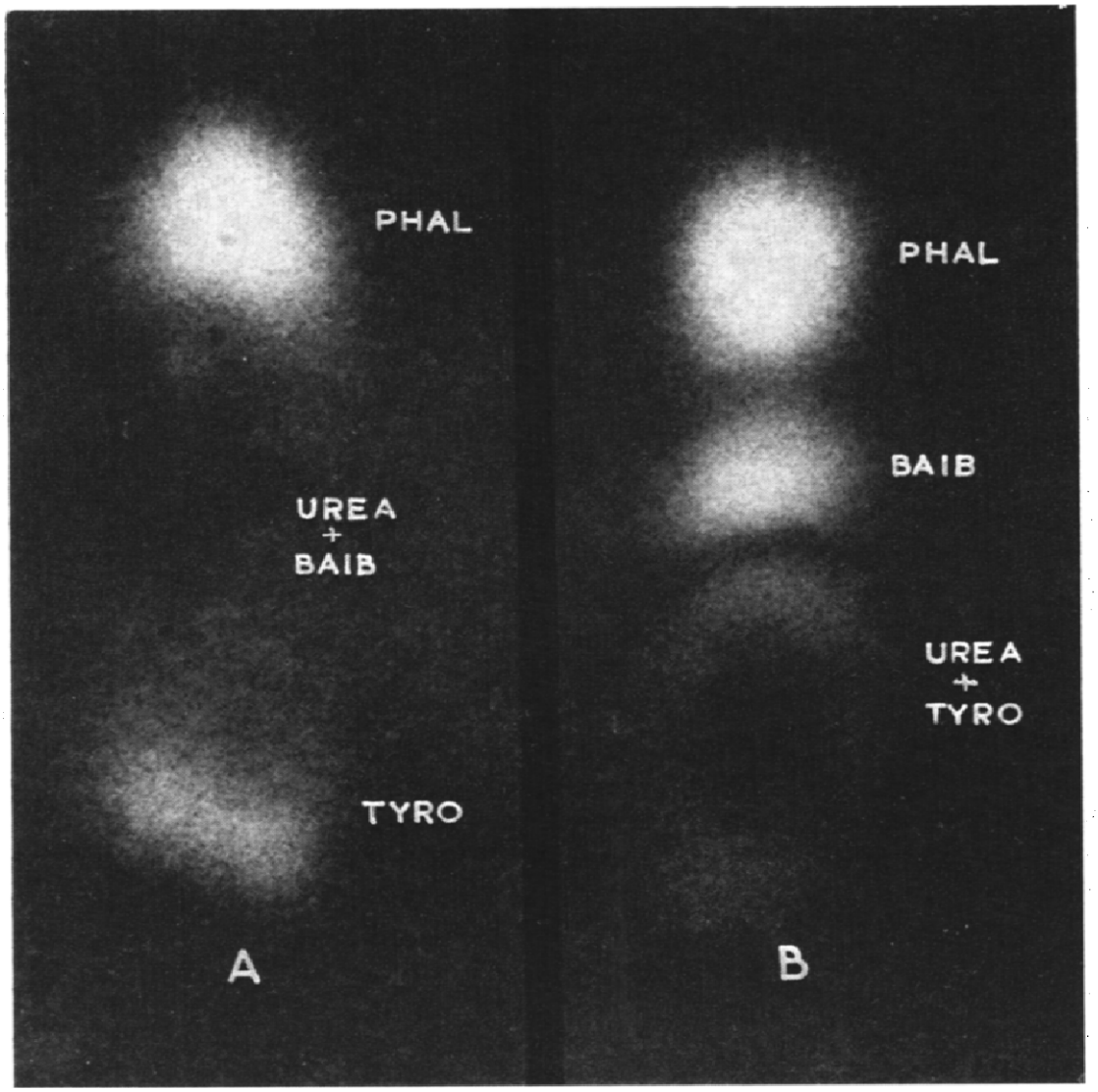

Fig. 1. Uni-dimensional (ascending) chromatogram showing the separation of phenylalanine, β-aminoisobutyric acid and tyrosine in the presence of excess of urea in runs of 12 h. (A) Separation in the solvent *n*-butanol–acetic acid–water (12:3:5). (B) Separation in the solvent isopropanol–*n*-butanol–isoamyl alcohol–formic acid–water (5:3:2:1.5:2).

(R_F values 0.46 and 0.52, respectvely). It has also been observed that the BAIB spot is more compact in the presence of urea than in its absence, suggesting a displacement effect because of urea. The use of this solvent, in uni-dimensional separation of BAIB, therefore, makes any special efforts to remove urea from the sample unnecessary.

In a study of urinary excretion of BAIB in 87 individuals, using the method and the solvent described under *Experimental*, there was no difficulty in labeling the chromatograms as BAIB negative in 60 cases. The remaining 27 cases appeared to be positive, but in a number of instances it was not possible to be certain whether the spot in question was really that of BAIB or of some other amino acid. This led us to restudy all these 27 cases by using two-dimensional chromatography, and it was interesting to see that out of these 27 cases, only 12 proved to be BAIB positive.

The failure, in our hands, of the uni-dimensional technique, often recommended for the study of urinary BAIB, might be attributed to the following reasons:

(1) A certain percentage of our normal population excretes detectable amounts of methionine and/or valine in urine. R_F values of BAIB, methionine and valine are 0.52, 0.52, 0.56 and 0.49, 0.50, 0.51 (ref. 11), respectively, in the solvent of the present study and the solvent used by BAWDEN[10]. These amino acids when present will, therefore, always cause confusion in the identification of BAIB.

(2) The method of identification of BAIB in uni-dimensional chromatography is based on the property of the usual amino acids to react with ninhydrin in the cold and inability of BAIB to do so. It may be stated, however, that at a room temperature* of 37°, BAIB shows almost full color development in 24 h. Even at other temperatures, there is always an overlap between time taken by α-amino acids for full color development and time taken by BAIB to show some detectable color.

Thus, in our opinion, uni-dimensional chromatography cannot be regarded as an ideal procedure for the study of urinary BAIB, until a solvent with the following two properties is known (a) in which separation is not influenced by the presence of urea; (b) in which the R_F value of BAIB is appreciably different from that of tyrosine on one hand and methionine and valine on the other hand.

In the opinion of the present authors, a fairly simple and accurate method for paper chromatographic study of urinary β-aminoisobutyric acid is the one recently worked out by SAINI[13]. In the method, untreated urine (≡ 40 μg creatinine) is applied in one corner of a 35 × 28 cm sheet of filter paper, Whatman No. 1. First run (overnight) is given along the length of the paper in the solvent isopropanol–water–formic acid (18:2:2). This helps to remove amino acids like leucine, isoleucine, phenylalanine, methionine, valine, tryptophan, BAIB, tyrosine, alanine and proline, as discrete spots, away from the influence of interfering substances, for any subsequent runs. This is followed by a second run (4-h) along the breadth of the paper in the solvent acetone–pyridine–ammonia (5:3:2) (ref. 14) and a third run (overnight), again along the length of the paper in the solvent isopropanol–*n*-butanol–isoamyl alcohol–formic acid–water (5:3:2:1.5:2) (see Fig. 2). The solvent for the second run, mentioned above, may be replaced by any other suitable solvent, but the solvent for the third run has to be such that it is not adversely affected by the partial separation achieved under the influence of the first "desalting" run. This method is suitable for the study of any of the amino acids referred to above.

In conclusion it may be stated that in our experience, uni-dimensional chroma-

* Our room temperature remains about 37° over a long period in summer.

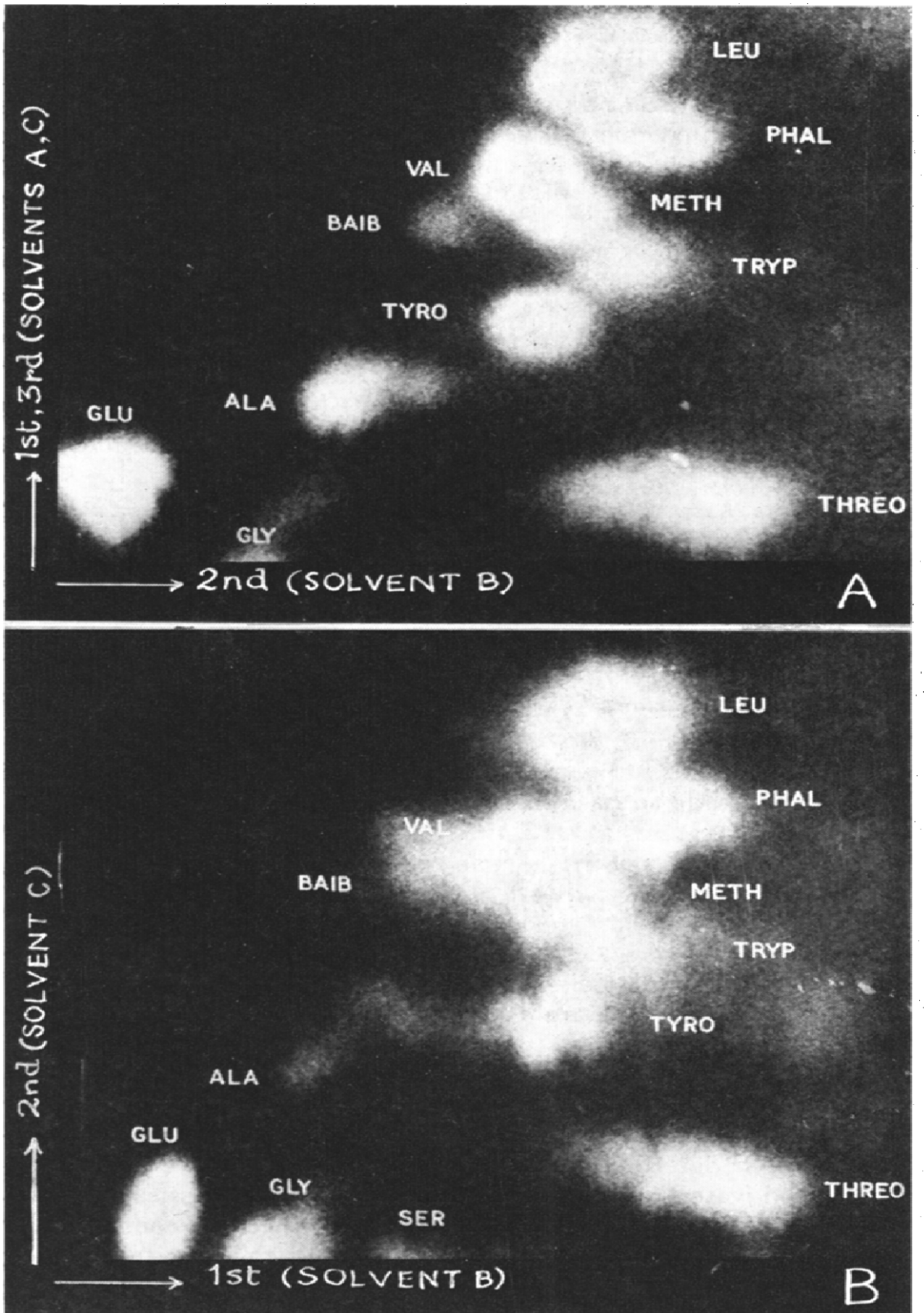

Fig. 2. Segments from two-dimensional (ascending) chromatograms illustrating the "desalting effect" of an initial overnight run in the solvent isopropanol–formic acid–water (18:2:2) for certain amino acids in the method of SAINI[13]. A standard solution containing all the amino acids seen in the chromatograms, except alanine and glycine, was first applied followed by application of an untreated urine ≡ 40 μg creatinine on the same spot. Solvent A: isopropanol–formic acid–water (18:2:2), 12-h run. Solvent B: acetone–pyridine–ammonia (5:3:2), 4-h run. Solvent C: isopropanol –*n*-butanol–isoamyl alcohol–formic acid–water (5:3:2:1.5:2), 14-h run. Abbreviations for amino acids are as follows: LEU = leucine, PHAL = phenylalanine, VAL = valine, TRYP = tryptophan, TYRO = tyrosine, ALA = alanine, METH = methionine, BAIB = β-aminoisobutyric acid, GLU = glutamic acid, GLY = glycine, THREO = threonine. (A) Chromatogram for which the first desalting run in solvent A is given; (B) chromatogram for which the run with solvent A is omitted.

tography is not satisfactory for isolation of urinary BAIB. The method of SAINI[13], although utilizing three runs, appears to be the method of choice, since it avoids losses and inconvenience of the desalting procedure.

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Separation of growth regulators from *Picea abies* Karst. on Sephadex LH-20

The occurrence of endogenous growth regulators in plants is usually investigated by means of various biological assays. The separation of the active substances from substances interfering with their determination is an important problem. Partition between organic solvents and water[1] and paper chromatography[2] are classical methods which have been used for this purpose. Some plant species, especially conifers, contain large quantities of growth inhibitors which can be difficult to separate from the auxins if only these methods are used[3,4].

The results reported here were obtained during an investigation of endogenous growth regulators in sprouting buds and seedlings of Norway spruce (*Picea abies* Karst.) The plant material was frozen and extracted with cold methanol. The *acid ethyl ether fraction* obtained by conventional methods[1,5] and a *butanol fraction* obtained by extraction of the aqueous acid solution, remaining after the ether extraction, with *n*-butanol were used in the experiments. The Avena straight-growth coleoptile test was used to detect biological activity.