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Note

Determination of amino acid enantiomers by two-column gas chromatography with valveless column switching

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Amino acids in serum are usually determined by the classical ion-exchange procedure first described in 1958 by Spackman *et al.*[1]. The application of chromatographic methods to the determination of amino acids has developed rapidly in recent years. Although most studies have employed liquid chromatography, thin-layer chromatography and paper chromatography[2], gas chromatography (GC) is attractive due to its high sensitivity, speed and the ability to separate enantiomers. Since the development of glass capillary columns coated with chiral silicone phases[3] and the concept of enantiomer labelling[4], GC has become a most powerful technique for amino acid analysis in biological fluids.

Gas chromatography based on valveless column switching is still in its development stage. Ten years after the introduction of this switching technique by Deans[5], commercial instruments enabling solvent-cutting, heart-cutting and backflush are now available. The analysis of amino acids in serum by GC has the shortcoming that large quantities of solvent or high boiling impurities in the sample can reduce the life of the column. Using the switching technique, the solvent and the high boiling impurities can be diverted from the pre-column. This not only increases the life of the main column, but also decreases the analysis time, as no conditioning of the column at high temperature is required.

Moreover, the use of a pre-column enables complete separation of a complex sample. For example, there are twenty L-amino acids in serum giving rise to 39 peaks for all their enantiomers when analysed on a optically active column. In this case, some peaks of different amino acids, *e.g.*, *allo*-Ile, Ile and Gly, are usually eluted close together or even overlap. Such difficulties can be overcome by using the column switching technique.

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In general, the separation of a complex sample depends mainly on the selectivity of the column; some of the amino acid enantiomers are eluted in different orders depending on the column temperature. The ideal separation of amino acids can be achieved by selecting carefully the rate of temperature programming for both pre-column and main column.

Another advantage is the reasonable analysis time. The purpose of amino acid analysis in serum is often the diagnosis of disease states. For example, in an investigation of the relationship between phenylalanine or tyrosine and cancer cells it is not necessary to determine all amino acids. Instead, only phenylalanine or tyrosine is introduced into the main column while the other amino acids are flushed back or passed to a different detector. Only the compounds of interest enter the main column and appear on the chromatogram.

EXPERIMENTAL

Preparation of samples

The method of sample preparation including preparation and treatment of blood, derivatization of pure amino acids and L-amino acids derived from serum was as described by Frank *et al.*[6].

Preparation and treatment of blood. Blood drawn from a pregnant woman was centrifuged to remove all blood particles. Then 0.5 ml of the serum were mixed with 1 ml of 1% picric acid with vigorous stirring to precipitate protein. The precipitate was washed with 1 ml of the picric acid. After centrifugating, the supernatant was passed through column (250 × 8 mm I.D.) packed with 1 ml of Type H ion-exchange resin, Dowex AG 50-X8 (100–200 mesh). After washing with 20 ml water, 8 ml of aqueous ammonia followed by 8 ml of water were passed through the column to elute all amino acids. The combined eluate was evaporated in a vacuum drying oven (below 50°C) to dryness.

*Derivatization of amino acids.*The residue obtained above was dissolved in 0.5 ml of *n*-propanol containing 4 *M* HCl and mixed by ultrasonication for 3 min. The solution was sealed in an all-glass tube and esterified in a water-bath at 100°C for 30 min. It was then dried with a gentle stream of nitrogen at room temperature. Methylene chloride (100 μl) and 100 μl of pentafluoropropionic anhydride were added to the residue. Then the ampoule was sealed again and heated to 150°C for 10 min. After cooling, the solution was dried with a gentle stream of nitrogen. The residue was dissolved in methylene chloride and analyzed by GC.

Apparatus and analysis conditions. The instrument used was a Sichromat 2 gas chromatograph (Siemens). This is a two-column GC system with two separate ovens (Fig. 1). The columns are connected with a T-type conrector. The two flame ionization detectors, D1 and D2, monitor the pre-column and the main column, respectively. Column 1 is a glass capillary coated with OV-101; column 2 is a glass capillary coated with Chirasil-val stationary phase. There are six steps of temperature programming for the two ovens. The functions of solvent-cutting, heart-cutting and backflushing are controlled by adjusting the valves NV1, NV2 and NV3, and automatic switching of magnetic valves MV1 and MV2. Hydrogen was used as carrier gas.

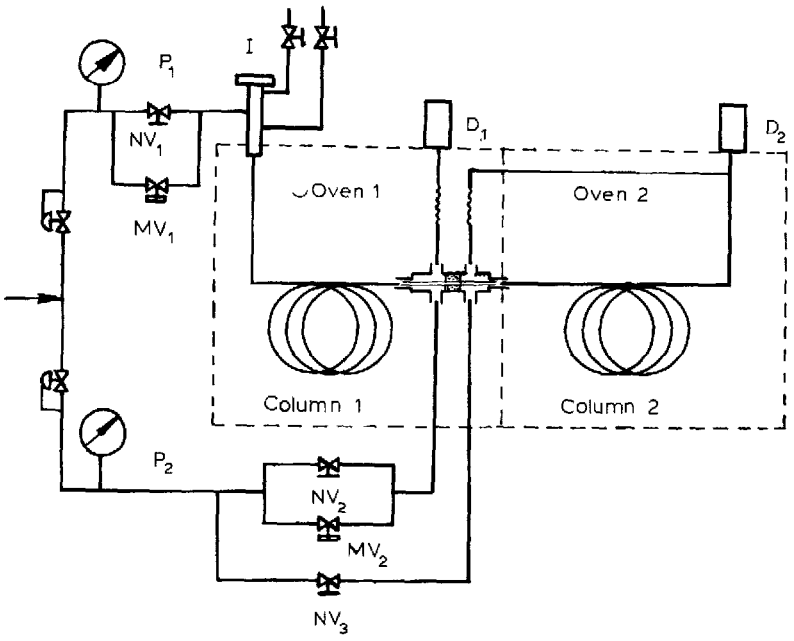

Fig. 1. Two-column GC system with valveless switching technique. I = injector; P_1 = inlet pressure of pre-column; P_2 = inlet pressure of main column.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of pure amino acids where the solvent peak was diverted from the main column by solvent-cutting. Fig. 3 shows the chromatogram of the same amino acid sample but with a different rate of temperature programming. The analysis times and elution orders of some amino acids are different in the two chromatograms.

In order to determine qualitatively the L-amino acids, serum was treated and analyzed in the same manner. By comparing chromatograms (see Fig. 4), L-amino acids in serum were easily identified by their retention times.

The separation of amino acids is affected by several factors. First, the selectivity of both the pre-column and main column play an important rôle. Table 1 lists the elution orders of amino acids on different columns. The order of elution from the two-column GC system differs bnetween OV-101 and the chiral phase. On the non-polar polydimethylsiloxane OV-101 the order is mainly determined by the volatility of the amino acid derivatives, whereas Chirasil-val exhibits considerable hydrogen-bonding properties[7], both stereoselective and non-selective. Additional hydrogen-bonding groups in the amino acid derivatives generally retard the elution. The elution order is also dependent upon the relative shielding by bulky groups, *e.g.*, Thr/Ser, and the fit between the stationary chiral selector and the respective selectand, *e.g.*, Ile/Leu. Finally, from Figs. 2 and 3 it is seen that the order of some amino acids is dependent on the temperature program. This means that the separation of amino acids can be optimized by changing the rate of temperature programming for the pre- and main columns.

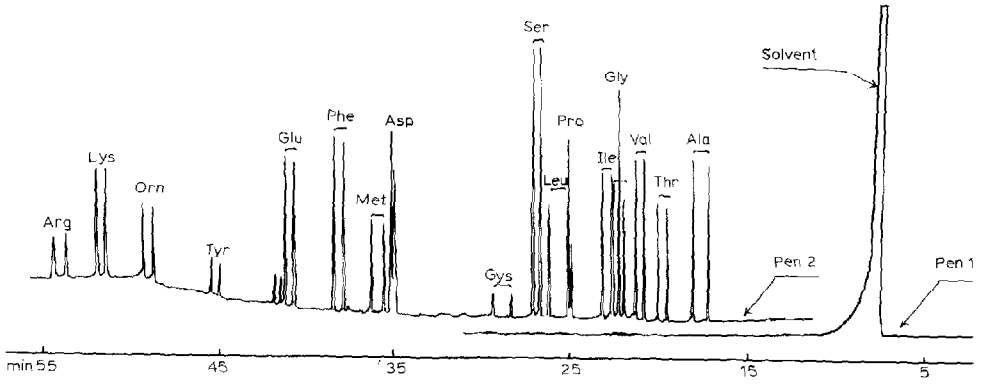

Fig. 2. Chromatogram of amino acid enantiomers obtained by two-column GC. Column temperatures: 1, from 40 to 200°C at 8°C/min; 2, from 50 to 200°C at 3°C/min.

Fig. 5 shows a chromatogram of nine amino acids which are usually eluted close together or overlap. Different elution orders can be obtained by use of different rates of temperature programming. For example, the separation of Leu and Pro, and of a-Ile and Ile, is not satisfactory in Fig. 5A, while in Fig. 5B only Ile and a-Ile are not separated completely. The elution orders of these amino acids at different rates of temperature programming are shown in Table II. The main difference between the two programs is a lower and flatter temperature gradient in case B for column 1. Consequently the longer residence time of the derivatives in this column leads to an elution order more strongly influenced by the non-polar *OV-101*. If quantitation of only a few selected amino acids is required, considerably shorter analysis times can be achieved.

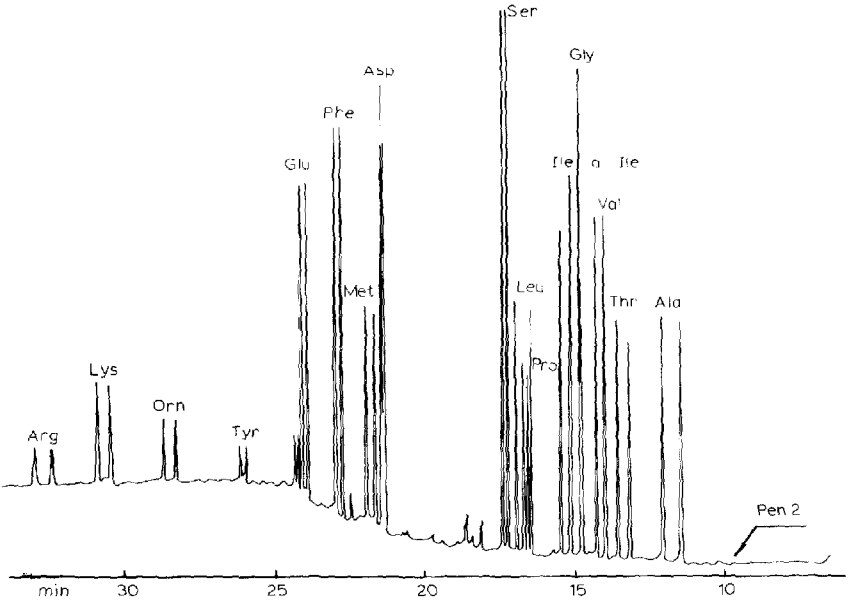

Fig. 3. Chromatogram of amino acids in Fig. 2 obtained with a different temperature programming: column 1, from 80 to 200°C at 10°C/min; 2, from 15 to 100°C at 2.5°C/min then at 7°C/min to 200°C.

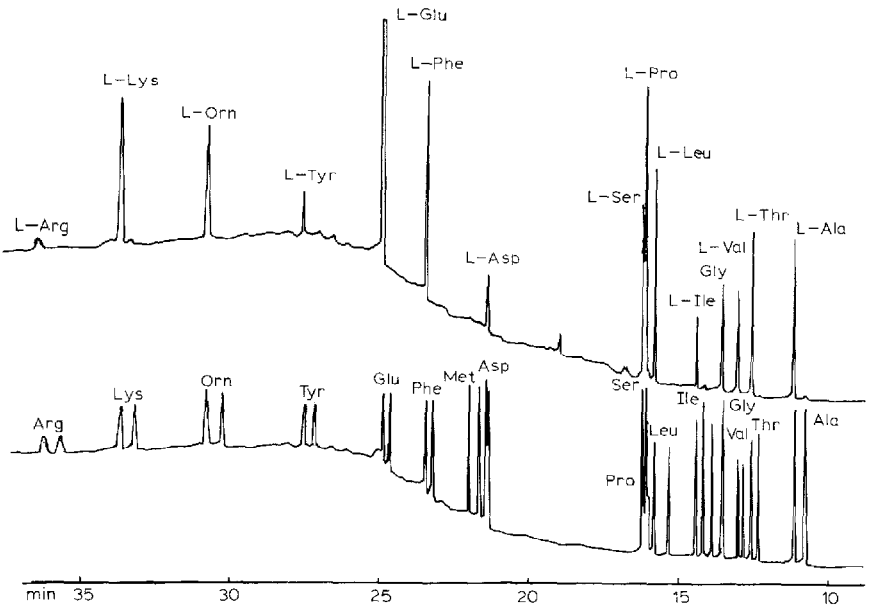

Fig. 4. Comparison of a chromatogram of L-amino acids in serum and a standard of D,L-amino acids. Column 1, from 80 to 200°C at 10°C/min; 2, from 75 to 200°C at 5°C/min.

TABLE I

ELUTION ORDERS OF AMINO ACIDS ON DIFFERENT COLUMNS

Amino acid	*Elution order*		
	OV-101	*Chiral*	*OV-101 and chiral**
Ala	1	1	1
Gly	2	5	2
Val	3	2	4
Thr	4	3	3
Ser	5	9	7
Leu	6	8	8
a-Ile	7	4	6
Ile	8	6	5
Pro	9	7	9
Cys	10	11	10
Met	11	12	12
Asp	12	10	11
Phe	13	13	13
Glu	14	14	14
Lys	15	17	17
Tyr	16	15	15
Orn	17	16	16
Arg	18	19	18
His	19	18	–
Trp	20	20	–

* Column temperature: pre-column, 80°C (Isothermal for 5 min) raised at 10°C/min to 130°C then at 15°C/min to 200°C; main column, 75°C raised at 5°C/min to 180°C.

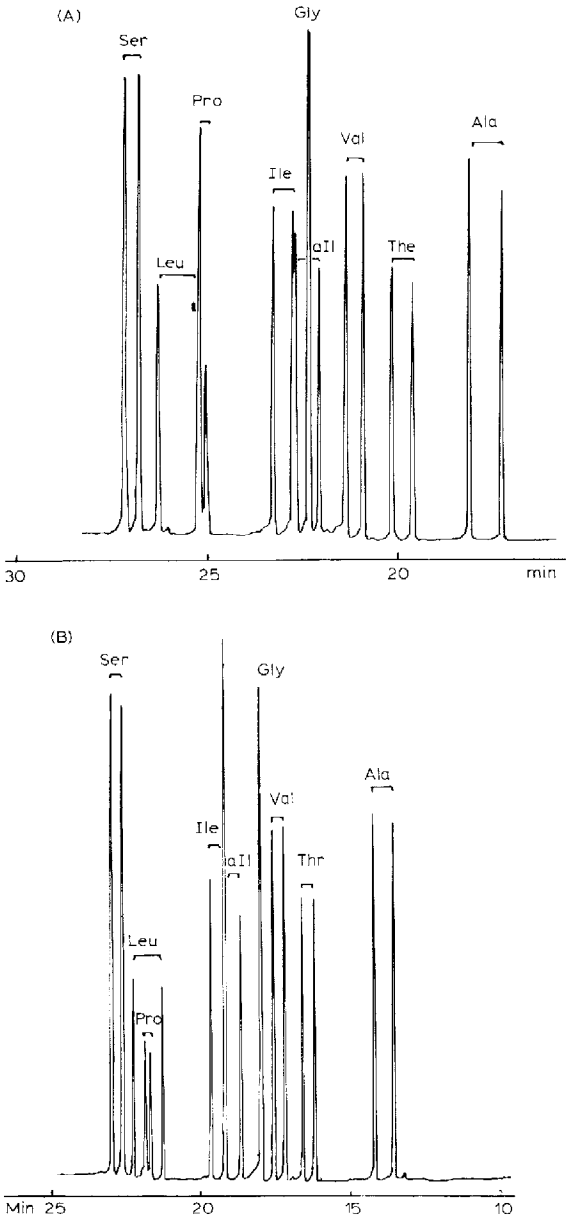

Fig. 5. Chromatograms of nine amino acids obtained with different temperature programs (see Table II).

The separation of L and D enantiomers is satisfactory except for Asp and Pro, which depends on the chiral column and its temperature. Fig. 6 shows chromatograms obtained by heart-cutting: A and D show the elution orders of nine amino acids on the pre-column and main column respectively, peaks 4, 5 and 8 were cut out (B), and introduced into the main column (C).

In conclusion, good separation of all amino acids in serum can be obtained.

TABLE II

EFFECT OF RATE OF TEMPERATURE PROGRAMMING ON ELUTION ORDER OF AMINO ACIDS

Amino acids	*Temperature programming rate**	
	A	*B*
Ala	1	1
Gly	5	4
Val	3	3
Thr	2	2
Ser	9	9
a-Ile	4	5
Ile	6	6
Leu	8	7
Pro	7	8

* A, Column 1, from 80 to 200°C at 10°C/min; column 2, from 75 to 100°C at 2.5°C/min, then at 7°C/min to 200°C. B, Column 1, from 40 to 200°C at 8°C/min; from 75 to 120°C at 2.5°C/min, isothermal for 0.1 min, then at 3°C/min to 200°C.

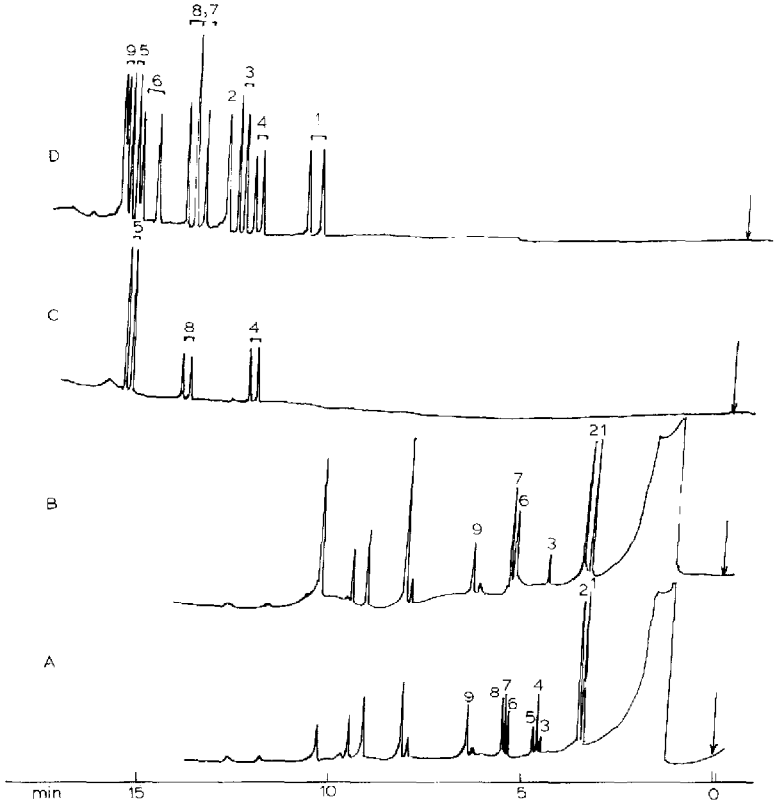

Fig. 6. Improvement of the separation of amino acids by use of the column switching technique: A, elution order of amino acids on pre-column; B, amino acids 4, 5 and 8 cut out; C, amino acids 4, 5 and 8 eluted from main column; D, elution order of amino acids on main column. Peaks: 1 = alanine; 2 = glycine; 3 = valine; 4 = threonine; 5 = serine; 6 = leucine; 7 = a-isoleucine; 8 = isoleucine; 9 = proline.

This is not always necessary; sometimes only one or several pairs of enantiomers are of interest in clinical studies. The analysis time for all twenty amino acids is about 30–60 min depending on the particular program.

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