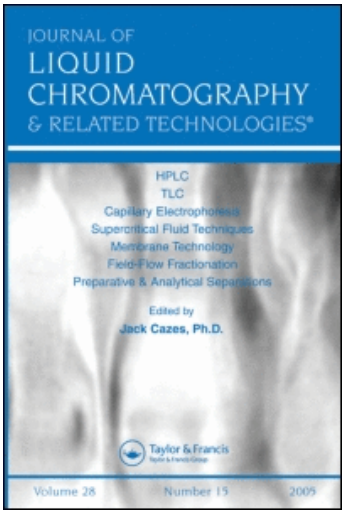

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Ion-Exchange Chromatography and Ion-Pair Chromatography. Complementation of HPLC Analysis of Amino Acids in Body Fluids by Pre-Column Derivatization Using Ortho-Phthaldialdehyde

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ION-EXCHANGE CHROMATOGRAPHY AND ION-PAIR CHROMATOGRAPHY. COMPLEMENTATION OF HPLC ANALYSIS OF AMINO ACIDS IN BODY FLUIDS BY PRE-COLUMN DERIVATIZATION USING ORTHO-PHTHALDIALDEHYDE

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Abstract

The determination of free amino acids in some human body fluids using reversed phase high performance liquid chromatography and pre-column derivatization with ortho-phthaldialdehyde/mercaptoethanol is a highly sensitive and reproducible method. However not all amino acids can be determined using this easily automatizable method. Several special amino acids, as for example cystin which contains sulphur, can be determined using a separation procedure of ion-exchange and/or ion-pair chromatography. These separation procedures are described here.

Introduction

A variety of amino acids and different concentrations of these are to be found in various body fluids such as blood, urine, cerebrospinal fluid and in aminiotic fluid during pregnancy [1]. The concentration of the single amino acids depends upon sex, age and circadian rhythm and feeding and is therefore subjected to continual changes. In addition to these normal changes in the concentration of the amino acids, pathological changes can also occur, for example due to metabolic diseases and deficiency symptoms. Within the field of perinatal medicine the diagnostics can be supported by detecting pathological changes and these can be markers of certain insufficiencies [1].

High performance liquid chromatography (HPLC) is particularly well suited to quantitatively determine a number of amino acids and similar substances in biological samples parallel in a single analysis [1-3].

The separation procedure still used today, which has been superior over a longer period of time, is ion-exchange chromatography (IEC) [4]. Since direct detection is extremely difficult or very insensitive in many of these substances, a post-column derivatization with good chromophores or fluorescence-active substances was connected to the separation online. At the end of the 1970s experiments were also made with ion-pair chromatography (IPC) using post-column derivatization [4,5] even when this separation procedure was first described as a solvent-generated ion-exchange system or dynamic ion-exchange chromatography [4]. The separation procedure for amino acids used today is the reversed phase (RP) chromatography with pre-column derivatization [6,7]. Table 1 lists the pre-column and post-column derivatization methods.

Each of these derivatization or separation methods used has its advantages and disadvantages. A paper [23], which compares the four pre-column derivatization methods that are used most often, comes to the conclusion that favours the easily automatizable OPA procedure, despite its disadvantages, only primary amines and none containing sulphur. The two most important secondary amino acids, proline and hydroxyproline, can be determined through a combination of the derivatization with OPA and NBD in a second, 15 minute separation [26,27].

In this study two isocratic separation methods are described in detail, which were developed for the separation of arginine metabolites using ion-exchange [28,29] and ion-paired chromatography [30,31]. These methods are a good complement to the OPA and OPA/NBD procedure.

TABLE 1

Pre-Column and Post-Column Derivatization Methods for the Analysis of Amino Acids

Post-column derivatization	Pre-column derivatization
Fluorescamine [6]	6-Aminoquinolyl-N-hydroxysuccinimidylisocyanate, AQC [10-12]
Ninhydrin [6]	Diaminoazobenzeneisothiocyanate, DABTH [6]
ortho-Phthaldialdehyde [6,8]	Diethyl ethoxymethylenemalonate [13]
Sodium benzoxazole-2-sulfonate [6,9]	4-(Dimethylamino)azobenzene-4'-sulfonyl chloride, Dabsyl [6,7,11,14]
	1-Dimethylaminonaphthalene-5-sulfonyl chloride, Dansyl [6,7,11]
	Diphenylindonylisothiocyanate, ITH [6]
	9-Fluorenylmethyl chloroformat, FMOC [11,15]
	Fluorescamine [7]
	Fluorodinitrobenzene, DNP [6,7,16]
	7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole, NBD [6,11]
	Methylisocyanate, MTH [6]
	Naphthalene-2,3-dicarboxaldehyde, NDA [11,17]
	ortho-Phthaldialdehyde, OPA [6,7,11,18]
	Phenylisothiocyanate, PTH, PITC, PTC [6,10,11,19-21]
	Sodium benzoxazole-2-sulfonate [22]

Materials

Chemicals

The amino acid standards (listed in table 2) and the albumine from bovine were supplied by the firms Sigma Chemie GmbH, Deisenhofen, Merck, Darmstadt, and Fluka, Neu-Ulm, all from Germany. The derivatization reagents were supplied by Merck, Darmstadt, and Fluka, Neu-Ulm (both from FRG).

The buffer substances used (sodium dihydrogen phosphate, sodium acetate), acids (perchloric acid, boron acid, ortho-phosphoric acid, sulphuric acid and hydrochloric acid), salts (sodium chloride) and the basis used (sodium hydroxide) and also the ion-pair reagent (heptane

sulphonic acid sodium salt monohydrate) all came from the firms Merck, Darmstadt, and Fluka, Neu-Ulm, both FRG.

Methanol with purity grade pro analysis is a product of the firm Merck, Darmstadt, or the firm Fluka, Neu-Ulm, FRG. The "destilled" water used was tap water, since it has been found to be UV purer compared to the bought product. This water is only deionized.

Body Fluid Samples

The amniotic fluid was collected during labour. The blood samples were withdrawn from the umbilical cord after delivery. These samples were stored in a refrigerator over a short period of time or were placed uncentrifuged in a deep freeze at approx. -20°C.

HPLC Equipment

The apparatus for the RP chromatography with amino acids derivatized with OPA is made up from pieces of equipment acquired from the following producers: the ERC Gesellschaft für den Vertrieb wissenschaftlicher Geräte m.b.H., Alteglofsheim (degasser type 3510), "Gynkotek" Gesellschaft für den Bau wissenschaftlich-technischer Geräte mbH, Germering bei München (autosampler Gina 160, fluoresence detector type RF 1001 and a C-R3A integrator with keyboard, monitor and floppy disk drive) and Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin (programmer 50B, two pumps type 64.00, a 6-port automatic valve with selected use of either automatic or manual injection, an injection valve with built-in reed relay and a column oven with integrated control unit). The separation column used first was an OPA-special column with a coarseness of 5µm and dimensions of 250mm x 4.6mm from the firm Knauer and later a Nova-Pak C18 also with 5µm and 3.9mm x 300mm from Waters, Eschborn, all firms FRG. As a protection for these columns, pre-colums with dimensions of 10-20mm x 4mm filled with C18 resp. ODS were set up and were changed every week or every two weeks.

The modular apparatus for the ion-exchange chromatography consists of a pump model 300C from the firm Gynkotek, Germering, an injection valve, a column oven type 89.00 and a variable wavelength monitor type 87.00 from Knauer, Berlin, an integrator HP 3394 from the firm Hewlett Packard, Waldbronn, all FRG, and a flowmeter from Phase Separations Limited, Queensferry Clwyd, U.K. The columns used from the firm Knauer, Berlin, FRG, are filled with Nucleosil

100-5 SA. We either used two serially operated columns with dimensions of 30mm x 4.6mm and 250mm x 4mm (combination A) or two columns 250mm x 4mm (combination B).

The ion-pair chromatography apparatus contains a programmer 50B, a pump type 64.00, an injection valve and a column oven as column space from the firm Knauer, Berlin, a digital thermometer HP 2802A with PT 100 sensor from Hewlett Packard, Waldbronn, and a spectralphotometer UVD/160-2 and a C-R3A integrator with keyboard, monitor and floppy disk drive from the firm Gynkotek, Germering. We used either the separation column with a length of 250mm and an ID of 4mm filled with Eurospher 100-C8, 5µm supplied by Knauer, Berlin, or another column, 125mm in length, ID 4.6mm filled with spherisorb ODS II, 5µm supplied by Melz VDS GmbH, Berlin, all firms FRG.

Additionally "operation boxes" are included in the equipment for the RP and ion-pair chromatography, which were developed and constructed by the engineer from our working unit. An auto-zero function is built into these "operating boxes", which the detectors used do not have. Furthermore a pump control is integrated into the automatized apparatus, which switches the autosampler on hold when the column counter pressure sinks.

Other Equipment

A digital pH-meter from the firm Knick Elektronische Messgeräte GmbH & Co., Berlin, is used for measuring the pH with an electrode type 405 from Ingold Messtechnik GmbH, Steinbach im Taunus. The temperature controlling of the solutions is done with a thermostat type E3 from Haake, Berlin, and a Thermomix 1419 bath 4K from B. Braun, Melsungen AG, Melsungen.

The centrifuges used are a model Labofuge A with an angled rotor, 12 x 15ml glasses from the firm Heraeus-Christ, Osterode am Harz, and a model 3200 from Eppendorf Gerätebau Netheler+Hinz GmbH, Hamburg.

We used a spectrophotometer model 25 with matching recorder model 24-25ACC from Beckmann Instruments GmbH, München.

An ultrasonic bath Sonorex type RK 106 S from Bandelin electronic GmbH & Co. KG, Berlin, was used for homogenizing and degassing the eluents and solutions. For mixing the samples for preparing the injection solutions and for manual experiments of derivatization reactions, a mixer type 750.01 REAX 1 D from Heidolph-Elektro GmbH, & Co.,KG, Kelheim, likewise all FRG, was used.

Methods

Standard Solutions

All the standard solutions were deep frozen in portions to increase their durability and were stored at about -20°C. When there was a deposit volume of 10ml 400mg albumin as matrix and 90mg sodium chloride to improve the solubility later was added [27]. The concentrations of the single amino acids in solution 1 (table 2 shows the composition) - with the exception of ASN (250µmol/l), GLY (350µmol/l), MET (235µmol/l) and THR (210µmol/l) - lie within the range 185 to 200µmol/l. The composition of the concentrations in the three other solutions was selected according to the various UV activities at 268nm. The concentrations of the single amino acids are kept at almost the same in all the three solutions. The concentration of INO amounts to about 50µmol/l, ARG(NO) 40µmol/l, CYS 200µmol/l, HYT 80µmol/l, TRP 65µmol/l, L-NAME 50µmol/l, KYN 125µmol/l and ADE 20µmol/l.

OPA Method [24-27]

Before the samples can be derivatized they have to be deproteinized. As the derivatization takes place in the basic medium, deproteinizings with the addition of an acid [32] are unsuitable. The simple way is to add methanol, which can be added to HOS as "internal" standard [27]. 200µl of the sample, respectively 200µl of the dissolved, deep frozen standard solution is diluted with 800µl methanol, mixed and finally centrifuged with the Eppendorf 3200. Then 200µl are removed, either by manual or automatic derivatization, diluted with 80µl sodium borate solution pH 9.5 and 60µl OPA reagent (50mg OPA in 4.5ml methanol absolute with 0.5ml sodium borate solution pH 9.5 and 50µl 2-mercaptoethanol) and mixed. After a reaction time of about 3.5 minutes 25µl 0.5N hydrochloric acid is added as stop reagent and mixed together. Using the manual method an additional dilution of 1 to 4 is made with acetate buffer (eluent). It is not possible to do this with the autosampler Gina 160. The injection volume amounts to 20µl.

The column is heated to 40°C. The amino acids are separated by a flow of 1.0ml/min with a methanol gradient in sodium acetate buffer, 50mmol/l and pH 7.0. They start with 15% methanol. The gradient continues to increase linearly to 20% in 30 minutes, to 35% at 45 minutes and to 75% at 70 minutes. Then the methanol content remains constant for 5 minutes before it drops again to 15% within 5 minutes. The detector is regulated at 330nm excitation and 450nm emission. The duration of the chromatogramme lasts for 75 minutes.

OPA/NBD Method [26,27]

Preparation of the samples is the same as for the pure OPA method, that is, the same deproteinized solution can be used. Using a fresh deposit the methanol can contain the "internal" standard dehydroproline. 50µl of this deposit is mixed together with 250µl sodium borate solution and 50µl sulphur-free OPA solution (50mg in 4.5ml methanol and 0.5ml borate solution) and warmed at 60°C for 2 minutes. Then 25µl chlornitrobenzofurazan solution (50mg in 5ml methanol) is added, mixed and kept at a temperature of 60°C for 10 minutes. 120µl 0.5N hydrochloric acid are added to terminate the reaction. Then 20µl are injected.

The eluents used are the same as those used for the OPA method. The columns are also heated to 40°C. We start with 10% methanol at a flow of 1ml/min. The gradient increases linearly to 30% within 10 minutes, then a further 5% in the next 2 minutes, finally reaching 50% at 15 minutes. Then the methanol content is reduced to 10% within 5 minutes. A chromatogramme takes 20 minutes. The detector is regulated at 470nm excitation and 550nm emission.

Ion-Exchange Chromatography

This isocratic method was developed for the separation of arginine metabolites at room temperature with an eluent which contains 200mmol/l buffer and 10vol.% methanol with a pH of 2.3 [28,29].

The deep frozen standards are dissolved in 1ml water. For deproteinizing 250µl standard or sample solutions are added to 25µl approx. 4.5 molar perchloric acid, mixed and centifuged. 20µl are injected from the excess. The 30 minute separation is done isocratically with 25mmol/l, when not stated otherwise, sodium dihydrogen phosphate in water with 12.5vol.% methanol with a pH value of 2.3 at 35°C column temperature and 0.75 or 1.0ml/min according to the column combination. Detection was made at 268nm.

Ion-Pair Chromatography

This isocratic method was also developed for the separation of arginine metabolites [30,31]. The preparation of the sample is identical to that of the ion-exchange chromatography. The eluent is composed of water with 10 vol.% methanol and 18.5mmol/l heptane sulphonic acid sodium salt as ion-pair reagent. At room temperature a separation with a flow of 1.5ml/min lasts 30 (C8) or 45 to 60 (ODS) minutes. The UV detector is regulated at 268nm.

TABLE 2

Composition of the Standard Solutions of the Amino Acids

Amino acid	Abbreviation	Solution-No.			
		1	2	3	4
"Adenine"	ADE		X	X	
L(+)-Alanine	ALA	X			
L-2-Aminoadipic acid	AAD	X			
L-2-Aminobutyric acid	ABA	X			
3-Aminobutyric acid	b-ABA	X			
4-Aminobutyric acid	g-ABA	X			
L-Arginine hydrochloride	ARG	X			
L(-)-Asparagine	ASN	X			
L-Aspartic acid	ASP	X			
L-Citrulline	CIT	X			
L-Cystine	CYS	X	X	X	
3,4-Dehydro-DL-proline	DHP				
L-Glutamine	GLN	X			
L-Glutatic acid	GLU	X			
L-Glutathione	GSH	X			
L-Glycine	GLY	X			
L-Histidine hydrochloride	HIS	X			
L-Homoserine	HOS				
5-Hydroxy-L-lysine hydrochloride	HYL	X			
4-Hydroxy-L(-)-proline	HPR	X			
5-Hydroxy-L-tryptophan	HYT		X	X	X

Amino acid	Abbreviation	Solution-No.			
		1	2	3	4
Inosin	INO	X	X	X	
L(+)-Isoleucine	ILE	X			
L-Kynurenine	KYN		X	X	
L-Leucine	LEU	X			
L-Lysine monohydrocloride	LYS	X			
L-Methionine	MET	X			
N-Methyl-L-arginine acetate	ARG(ME)				
3-Methyl-L-histidine	HIS(ME)				
N-Nitro-L-arginine	ARG(NO)		X		X
N-Nitro-L-arginine methylester	L-NAME		X		X
L-Ornithine monohydrochloride	ORN	X			
L(-)-Phenylalanine	PHE	X			
ortho-Phosphoserine	P-SER				
L(-)-Proline	PRO	X			
L-Saccharopine	SAC	X			
L(-)-Serine	SER	X			
Taurine	TAU	X			
L(-)-Threonine	THR	X			
L-Tryptophan	TRP	X	X	X	
L(-)-Tyrosine	TYR	X			
L(+)-Valine	VAL	X			

TABLE 2

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		1	2	3	4
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L(+)-Alanine	ALA	X			
L-2-Aminoadipic acid	AAD	X			
L-2-Aminobutyric acid	ABA	X			
3-Aminobutyric acid	b-ABA	X			
4-Aminobutyric acid	g-ABA	X			
L-Arginine hydrochloride	ARG	X			
L(-)-Asparagine	ASN	X			
L-Aspartic acid	ASP	X			
L-Citrulline	CIT	X			
L-Cystine	CYS	X	X	X	
3,4-Dehydro-DL-proline	DHP				
L-Glutamine	GLN	X			
L-Glutatic acid	GLU	X			
L-Glutathione	GSH	X			
L-Glycine	GLY	X			
L-Histidine hydrochloride	HIS	X			
L-Homoserine	HOS				
5-Hydroxy-L-lysine hydrochloride	HYL	X			
4-Hydroxy-L(-)-proline	HPR	X			
5-Hydroxy-L-tryptophan	HYT		X	X	X

TABLE 2

Continuation

Amino acid	Abbreviation	Solution-No.			
		1	2	3	4
Inosin	INO	X	X	X	
L(+)-Isoleucine	ILE	X			
L-Kynurenine	KYN		X	X	
L-Leucine	LEU	X			
L-Lysine monohydrocloride	LYS	X			
L-Methionine	MET	X			
N-Methyl-L-arginine acetate	ARG(ME)				
3-Methyl-L-histidine	HIS(ME)				
N-Nitro-L-arginine	ARG(NO)		X		X
N-Nitro-L-arginine methylester	L-NAME		X		X
L-Ornithine monohydrochloride	ORN	X			
L(-)-Phenylalanine	PHE	X			
ortho-Phosphoserine	P-SER				
L(-)-Proline	PRO	X			
L-Saccharopine	SAC	X			
L(-)-Serine	SER	X			
Taurine	TAU	X			
L(-)-Threonine	THR	X			
L-Tryptophan	TRP	X	X	X	
L(-)-Tyrosine	TYR	X			
L(+)-Valine	VAL	X			

When the apparatus has not been in use the columns are previously washed with buffer and salt-free eluents, that is water or methanol/water.

Results and Discussion

OPA Method

The separation of standard solution 1 is illustrated in figure 1, as it is used for calibration. 28 of the 31 amino acids present can be detected. Additionally HOS was added as "internal" standard to the standard solution. Figure 2 shows a separation of the primary amino acids in amniotic fluid. Not all the substances present in the standard can be found. When the integrator is regulated to a higher sensitivity some small peaks are still visible, which could stem from AAD, g-ABA and HYL. Furthermore 7 peaks not assigned to the standard, can be found as unknown peaks. It cannot be ruled out that these are amino acids such as P-SER, ARG(ME) and HYT for example; but they could also be so-called biogenic amines or their derivates or other amino-functional substances. The concentrations of these substances, the 3 amino acids and the 7 unknown ones, must lie below 1µmol/l, since this is approximately - though it varies according to the specific substance - the threshold limit of the chromatogramme illustrated in figure 2.

OPA/NBD Method

Figure 3 shows the separation of the secondary amino acids according to the OPA/NBD method for the same samples illustrated in figures 1 and 2. Here an "internal" standard, dehydroproline, can also be included, although this is not absolutely necessary, since the reagent peak can be used as such. These chromatogrammes show several peaks which cannot be assigned to the standard and cannot be identified. Possibly a second proline peak and an arginine one can be seen.

Both these methods run automatically in so far as the sample apparatus performs the whole OPA derivatization and functions as automatic injector for the durable OPA/NBD derivates. Only when the GINA 160 apparatus is in action with the OPA method, there is the disadvantage that no further dilution can finally be performed, which not only shortens the life of the pre-columns but also that of the main column as well.

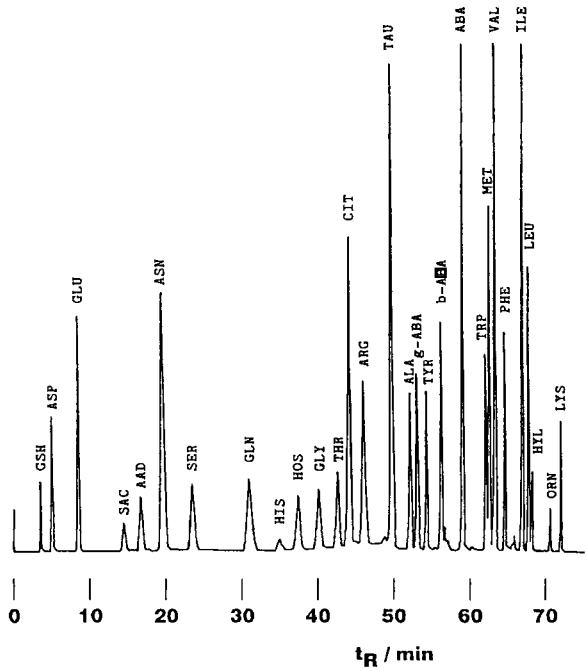

FIGURE 1: Chromatogramme of a manually OPA derivatized standard solution 1 on Nova-Pak C18, attenuation 512mV full scale, abbreviations see table 2 and t_R - retention time

With these two methods it is not possible to determine inosine or either of the amino acids cysteine and cystine which both contain sulphur.

Ion-Exchange Chromatography

The separation for arginine metabolites only, which was developed by Raberger and co-workers [28,29], is achieved with a buffer concentration of 200mmol/l and a pH of 2.3 at room temperature. The injection of an amniotic fluid sample produced several peaks which could not be assigned to the arginine metabolites. In addition to the arginine metabolites, ARG(NO) and L-NAME, 6 further amino acids could be detected. More detailed investigations and single injections of all of the 40 amino acids present, resulted in INO, CYS, HYT, TRP, KYN and ADE being found as well, whereby in actual fact adenine is not really an amino acid, but can occur in body fluids.

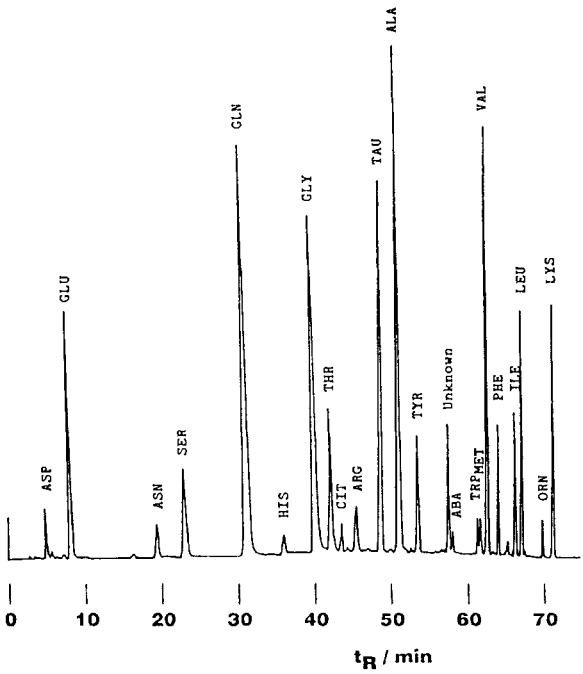

FIGURE 2: Separation of the primary amino acids of manually OPA derivatized, deproteinized amniotic fluid (No. 140) on Nova-Pak C18, attenuation 512mV full scale

On account of spectroscopic examinations in UV the concentrations in the standards were selected in such a way that the resulting peaks could possess approximately the same size peak surface, or that no single peak could be extremely dominant. For each of the 8 substances all of the same weight, extinctions were measured at 268nm - the detection wavelength - between 0.04AU (CYS) and 0.82AU (ADE). The molar extinction coefficients determined approximately from these measurements lie in the range of about 2000 to 25000 liter pro mol and centimeter.

Very soon it was apparent that the quality of the separation depends very much on the pH. A mere fluctuation of 0.05 to 0.1 in the pH level worsens the separation drastically. In our laboratory the room temperature changes quite considerably at times with a fluctuation of up to a maximum of 10°C, and therefore the pH level could not be held stable. The only way to compensate this and bearing in mind the apparatus at our disposal, was to keep the columns at a con-

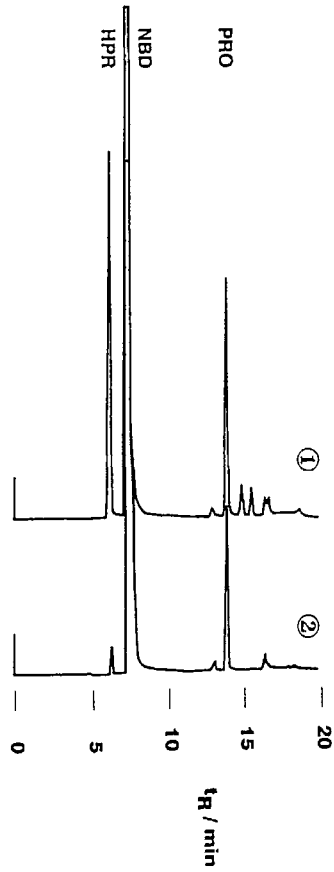

FIGURE 3: Separation of the secondary amino acids of an OPA/NBD derivatized standard solution 1 (①) and amniotic fluid (No. 140,②) on Nova-Pak C18, attenuation 16mV full scale, abbreviations see table 2 and t_R - retention time

stant temperature, in this case at 35°C. Figure 4 in three sets of measurements shows how the pH level for the eluent with 200mmol/l buffer and 10vol.% methanol is dependent on the temperature. As a result of this, the pH level of the eluent is regulated at the prevailing room temperature, which at 35°C gives a pH of 2.3.

The increase in temperature also caused a strong reduction in the retention times, so that the buffer concentration had to be decreased. Figure 5 shows the dependence of the retention

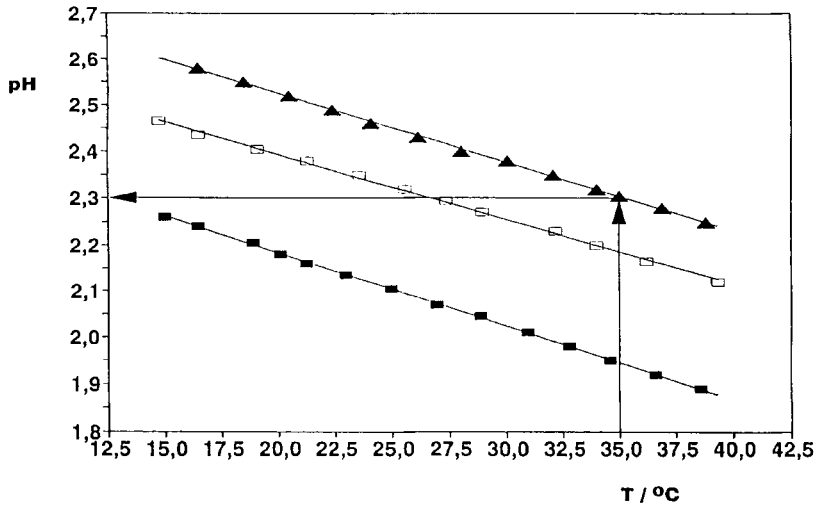

FIGURE 4: Temperature dependence of the pH level of the sodium dihydrogen phosphate solution, 200mmol/l in water with 10vol.-% methanol

times on the buffer concentration for column combination A. Next time and thereafter we used a buffer concentration of 25mmol/l. Furthermore we examined the influence of the methanol amount. Whilst in some of the substances the retention times are almost dependent on the amount of methanol, in others they decrease by up to 16% with an increase of the methanol content from 7 to 13%. In addition the width of the peak becomes smaller.

Figure 6 illustrates the chromatogrammes of the standard solutions 2 to 4. The chromatogrammes contain two intricate separation problems, as can also be seen in figure 5. These are the pairs of substances CYS/ARG(NO) and KYN/L-NAME which are not always separated. In this deposit used from standard solution 2, the results of the spectroscopic measurements were not yet included.

The columns used deactivate within the course of time. Figure 7 shows two chromatogrammes of standard solution 2, which were made under the same conditions at an interval of two months of each other. The retention times are reduced in all the substances and under three peaks in the second chromatogramme there are always 2 substances. We have not been successful in regenerating or reactivating the columns by rinsing with other fluids.

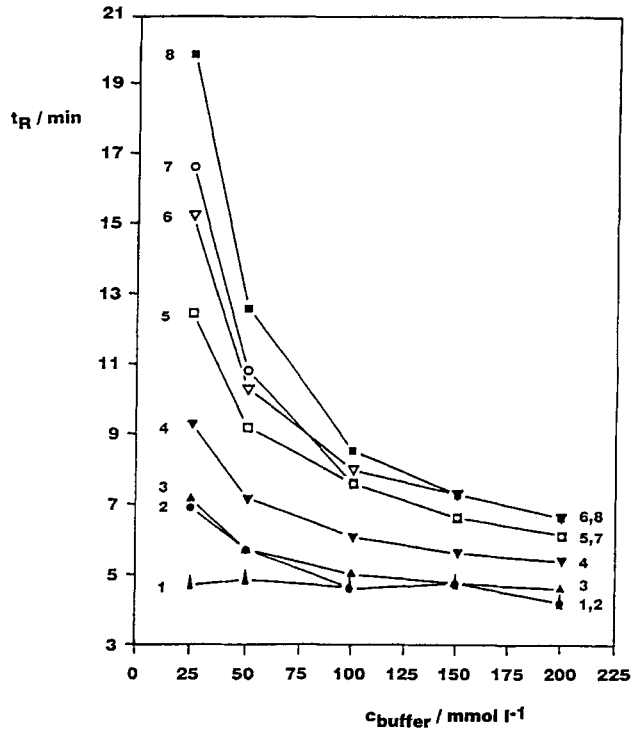

FIGURE 5: Dependence of the retention times t_R of the substances in standard solution 2 of the buffer concentration c_b of the eluent with 13vol.% methanol at 35°C;
1) INO, 2) CYS, 3) ARG(NO), 4) HYT, 5) TRP, 6) KYN, 7) L-NAME and 8) ADE

Figure 8 shows how the separation method is used on an amniotic fluid sample (No. 114) and an umbilical cord blood sample (No. 2). In the case of the amniotic fluid a clear assignment of TRP (11.45min) and CYS (6.25min) is possible. The peak at 4.3min could stem from INO without a sign whether only INO or other substances as well form this peaks. Similar situations occur with the blood sample. TRP (12.1min) and CYS (7.45min) can clearly be assigned over the retention time comparisons. All the other peaks cannot be assigned alone. These two chromatogrammes show characteristic peak distributions in the front retention time range for amniotic fluid - here 2 peaks up to 6 minutes - and umbilical cord blood - 5 peaks here up to 7

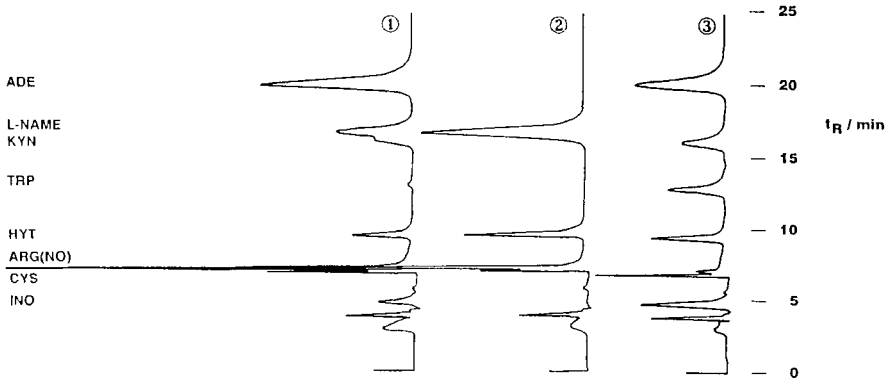

FIGURE 6: IEC: Chromatogrammes of the standard solution 2 (①), 4 (②) and 3 (③) on the column combination A, eluent 25mmol/l buffer in water with 11vol.% methanol, attenuation 64mV full scale, abbreviations see table 2 and t_R - retention time

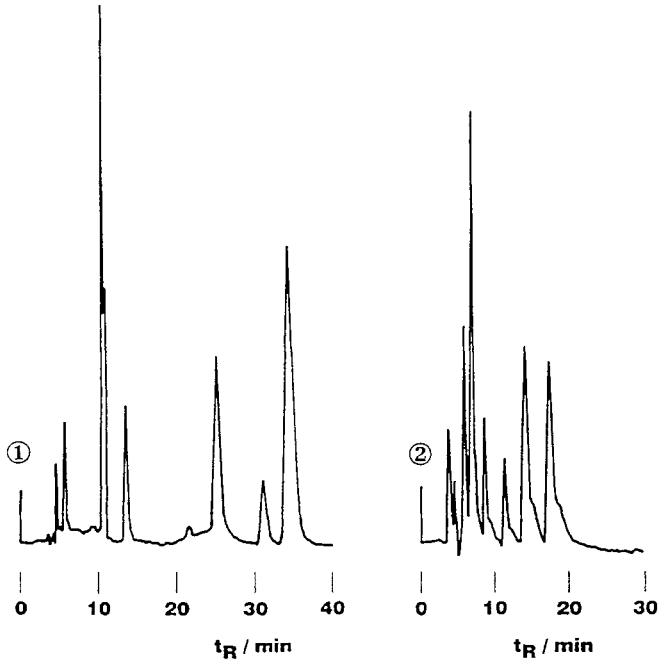

FIGURE 7: Deactivation of the ion exchange columns within 2 months (① ⇒ ②), with the example of the separation of standard solution 2 on combination B, eluent with 50mmol/l buffer and 12.5vol.% methanol, attenuation 64mV full scale

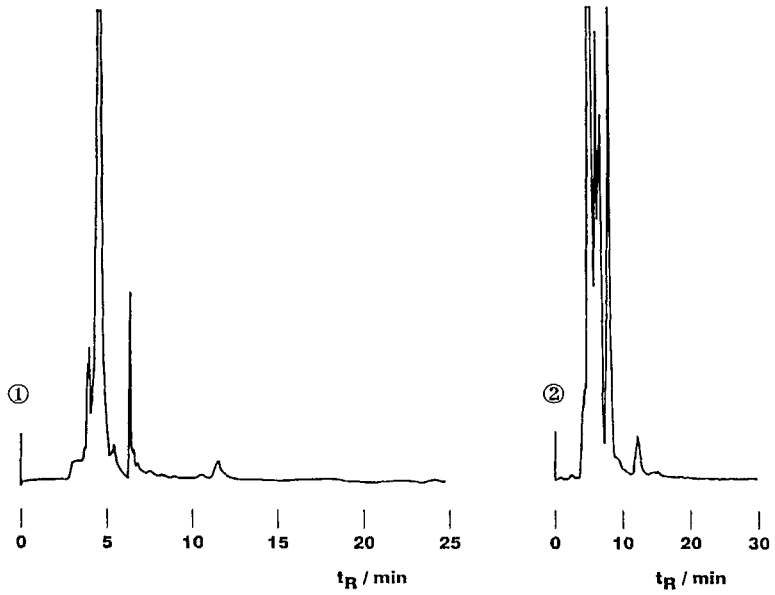

FIGURE 8: IEC: Chromatogramme of amniotic fluid (No. 114,①), combination A with 25mmol/l buffer with 12.5vol.% methanol and an umbilical cord blood sample (No. 2,②), combination B with 32.5mmol/l buffer and 12.5vol.% methanol, attenuation 64mV full scale

minutes. The above mentioned deactivating of the columns which is so fatal for proving the calibration hardly plays a part in this problem, for the substances are mostly present in such a very small concentration that nevertheless 2 peaks can be detected again. It is possible to make a quantitative evaluation according to the concentration when the peak is clearly identified. Difficulties only occur in the CYS peak as this represents two substances, dimeric cystine and monomeric cystein, which however produces an even smaller molar extinction coefficient. In the chromatogrammes of amniotic fluid and blood further peaks can occur which cannot be identified as amino acids, which is not otherwise to be expected when using mixtures containing so many different substances. In some of the amniotic fluid samples kynurenine was found in very small amounts. If the samples contain a lot of TRP then they can be compared with the quantitative determination using OPA.

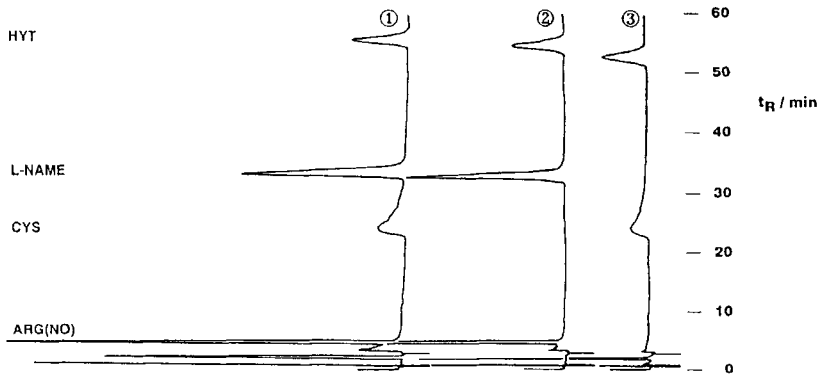

FIGURE 9: IPC: Chromatogrammes of standard solution 2 (①), 4 (②) and 3 (③) on the column Spherisorb ODS II, attenuation 16mV full scale

Ion-Pair Chromatography

The chromatogrammes of the 3 standard solutions from 2 to 4 on the Spherisorb ODS II column are shown in figure 9. With this method in addition to the arginine metabolites CYS and HYT can also be determined. The other substances achieve the peak at 2.1 minutes so that a quantitative evaluation is not possible. When the Eurospher C8 is in use there is no change in the succession of the retention, only the times are about half as long. With this method too, the columns are liable to deactivate after some time. In the case of the ODS columns the deactivation happens very much more quickly than in the RP8, as the three analyses performed after each other within three hours already show in figure 9. The retention time of HYT decreases by 2.2 minutes. In this case the deactivation is reversible. The columns can be reactivated again by rinsing with water, then methanol, then dichlormethane/tetrahydrofurane (1:1, v:v), methanol and water. Sometimes it is enough just to wash the equipment with water so that for example it can be switched off over night.

As in the ion-exchange chromatography, characteristic differences can be observed here too in the chromatogrammes for amniotic fluid and blood (see figure 10). These samples are identical with those in figure 8. A peak of cystine can clearly be seen in both samples. It is not possible to assign or identify the peak up to 5 minutes retention time. When the samples contain hydroxytryptophane it is then possible to check the concentrations determined with the IPC

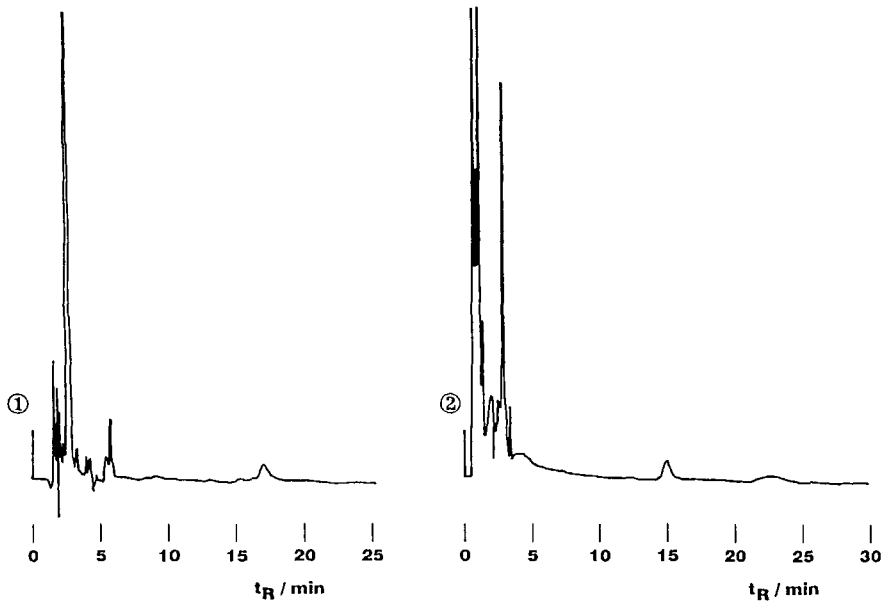

FIGURE 10: IPC: Chromatogramme of an amniotic fluid (No. 114,①), column Eurospher C8 and an umbilical cord blood sample (No. 2,②) on Spherisorb ODS II, attenuation 16mV full scale

method with the IEC method. In further measurements of amniotic fluid samples a discrepancy emerged in the concentrations determined for CYS using IEC and IPC. The concentrations determined with IPC were larger. A more detailed investigation showed that the peak assigned to cystine contains two substances. In addition to CYS, UV-activative tyrosine appears at this point as well. However, since TYR is determined using the OPA method, it is possible to calculate the concentration of cystine.

Both the methods, IEC and IPC, of determination of special amino acids are less sensitive due to UV detection at 268nm and have higher detection limits. Also peak assignment or identification is partially not possible. This aspect could be improved by using a diodenarray detector, since the few UV-active amino acids could then probably be identified through the spectra. If lower detection limits are required, then a post-column derivatization has to be used.

Conclusion

The two methods for determining special amino acids using IEC and IPC described here are suitable as an appropriate complement to the determination of amino acids with OPA and/or OPA/NBD. In this way in addition to the arginine metabolites, adenine and the other amino acids cystine, kynurenine, hydroxytryptophane and - to a limited extent - inosine and cystein can be determined. These methods are of interest when these amino acids do not have to be determined all the time, otherwise it is more appropriate to use another derivatization method for the determination of amino acids.

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