ELSEVIER

Journal of Chromatography A, 711 (1995) 201–215

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatographic determination of D- and L-amino acids by derivatization with *o*-phthaldialdehyde and N-isobutyryl-L-cysteine

Applications with reference to the analysis of peptidic antibiotics, toxins, drugs and pharmaceutically used amino acids

H. Brückner[a,*], T. Westhauser[a], H. Godel[b]

[a]*Institute of Food Technology, University of Hohenheim, 70593 Stuttgart, Germany*
[b]*Hewlett-Packard GmbH, Waldbronn Analytical Division, 76337 Waldbronn, Germany*

Abstract

In order to evaluate and extend the applicability of an analytical method that enables the quantitative and simultaneous high-performance liquid chromatographic determination of D- and L-amino acids (DL-AAs) by automated precolumn derivatization with *o*-phthaldialdehyde together with the chiral thiol N-isobutyryl-L-cysteine [J. Chromatogr., 666 (1994) 259] selected natural and synthetic bioactive peptides, as well as pharmaceutically used formulations of AA, were investigated and the amounts of D- and L-AA determined by fluorescence detection. Peptides containing cyst(e)ine were oxidized with performic acid prior to hydrolysis with 6 *M* HCl, and those containing Trp were hydrolyzed with 4 *M* methanesulfonic acid (24 h at 110°C in both cases). Peptides analyzed were the peptide antibiotics bacitracin, gramicidins A and S, polymyxin B, metanicin C, the peptide toxin malformin A and the peptide drugs D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, β-casomorphin and α_{s1}-exorphin. Further, the enantiomeric ratios of pharmaceutically used AA formulations containing racemic DL-Ser, DL-Asp and DL-Met were determined, and the AA drugs L-Asp and L-Trp were tested negatively for the presence of the respective D-enantiomers. In two aqueous formulations of L-AA used for parenteral nutrition, low amounts of D-AA (0.1–0.9% with respect to certain L-AA enantiomers and of totally 128 mg and 149 mg D-AAs per liter infusion solution) were determined.

1. Introduction

Since the discovery that α-amino acids (AAs) together with *o*-phthaldialdehyde (OPA) and thiols rapidly form highly fluorescent derivatives [1] which are separable by reversed-phase high-performance liquid chromatography (HPLC) [2], the method has been developed into one of the most widely used procedures for quantitative liquid chromatographic AA analysis [3–6]. With the use of laser-induced fluorescence detection this method also allows determination of AAs at subfemtomole levels [7].

As a result of intensive investigations on the

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00158-1

structure [8,9] and kinetics [10] of the isoindole derivatives formed, together with instrumental advances and an adequate derivatization chemistry, inherent shortcomings of the method were overcome. Thus, for example, the failure of the OPA–thiol reagents to react with secondary amino acids like Pro and Hyp was resolved by an additional derivatization with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) [3] or 9-(fluorenyl)methyloxycarbonyl chloride (Fmoc-Cl) [11,12], the low fluorescence yield of cysteine was enhanced by its conversion into carboxyethylthiocysteine by derivatization with 3,3′-dithiopropionic acid [13,14], and problems arising from the rapid decline of the fluorescence of Gly and Lys could be alleviated by highly reproducible, fully automated derivatization conditions.

The applicability of this analytical method for chiral AA analysis was shown by the observation that the reaction of DL-Asn with OPA and N-acetyl-L-cysteine (N-Ac-Cys) leads to the formation of diastereoisomeric *S*-[2-(1-carboxy-2-methylpropyl)isoindole-1-yl]-N-acetylcysteine derivatives which are separable by HPLC [15]. This elegant approach [15] was immediately applied to the separation of mixtures of protein L-AAs and their corresponding D-enantiomers [16–21], unusual natural DL-AAs such as D- and L-lombricine (D- and L-guanidinoethylphospho-O-serine) [22] and non-proteinogenic α-alkyl-α-DL-AAs [23–27]. OPA and N-Ac-Cys were also found to be suitable for the derivatization and liquid chromatographic resolution of the racemate of the potentially antiepileptic synthetic AA drug (*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid [28].

The possibilities for the replacement of N-Ac-Cys (also designated as NAC in the literature) by other chiral thiols such as *tert.*-butyloxycarbonyl-L-cysteine (Boc-Cys) [17] or N-acetyl-L-penicillamine (NAP) [20] were also realized. Thus, OPA together with NAC, NAP or Boc-Cys was used for the resolution of enantiomers of AAs and α-amino alcohols released in the course of total hydrolysis of the synthetic peptide drugs octreotide and [D-Cys^2]-octreotide [20] and for separating neuroactive non-protein AAs, viz. racemic 2-amino-ω-phosphonoalkanoic acid homologues and racemic α-amino-β-N-methylaminopropanoic acid [29] or the enantiomers of homologeous series of ω-N-oxalyldiamino acids [30]. Further, the resolution of diastereomers obtained by derivatization of (*R,S*)-α-alkyl-α-AAs with NAC and Boc-Cys was compared to those of compounds derived from derivatization with N^2-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide ("Marfey's reagent") [26].

Notably, the use of chiral thiol sugars as derivatizing reagents for DL-AAs has also been reported, e.g. 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside (TATG) [31] or the sodium salts of 1-thio-β-D-glucose [32], 1-thio-β-D-mannose [33] or 1-thio-β-D-galactose [34]. The reagents TATG, 1-thio-β-D-glucose (as well as NAC and NAP) have been used for the resolution of racemic amines used as pharmaceuticals [35].

The general applicability of the method for the resolution of DL-AAs has also been shown with the chiral thiols (3*S*)-neomenthyl thiol [36], (2*S*)-2-methyl-3-mercaptopropanol [37], 1-[(2*S*)-3-mercapto-2-methylpropanoyl]-L-proline ("Captopril") [38] or D-3-mercapto-2-methylpropionic acid [39]. It is also of interest in this context that micellar electrokinetic chromatography and capillary zone electrophoresis, respectively, were used for the separation of diastereomeric isoindole derivatives derived from DL-AAs, OPA and N-Ac-Cys or N-Boc-Cys [40–42].

Owing to the relatively easy accessibility of N-substituted derivatives of L-Cys, homologous series of N-acyl-L-cysteines (acyl = series acetyl to *n*-octanoyl, isobutyryl, isovalerly, pivaloyl and benzoyl) were synthesized [38,43–47] and systematically investigated for their abilities to separate single pairs or complex mixtures of DL-AAs as diastereomeric isoindole derivatives.

Among the various reagents tested for their suitability for separating multicomponent mixtures of DL-AAs, N-isobutyryl-L-cysteine (IBLC) and its enantiomer N-isobutyryl-D-cysteine (IBDC) [44,48–51] were selected as the most promising reagents. A straightforward liquid chromatographic method was developed which allows the complete separation of all protein L-α-AAs and their corresponding D-enantiomers,

as well as the achiral Gly and the internal standard L-*homo*-Arg [48] (Pro and other α-imino acids do not react with OPA–thiols).

Using a fully automated instrument with fluorescence detector, together with a standard octadecylsilyl stationary phase and simple linear gradient elution conditions, the method proved to be highly reproducible, sensitive and very robust. This was recently documented by numerous applications in the fields of biosciences [48] and food sciences [46,49–51].

In continuation of preliminary investigations on certain peptide hydrolysates [52], in this paper we demonstrate that the OPA–IBLC method is also highly suitable for the simultaneous quantification of L- and D-AAs in hydrolysates of natural and synthetic peptides, for the determination of the enantiomer ratio in drugs containing racemic AAs, and for the detection and quantification of minor amounts of D-AAs which might be present in pharmaceutical formulations of L-AAs used for parenteral nutrition.

Furthermore, we show that the enantiomers of cysteine and cystine are determinable by the method as cysteic acid after prior oxidation of the respective peptides with performic acid [3,53], and that hydrolysis of the substrates with 4 *M* methanesulfonic acid [3] makes possible the determination of acid sensitive D- and L-Trp in peptides.

2. Experimental

2.1. Chemicals

Methanol was of LiChrosolv grade, and acetic acid (100%), hydrogen peroxide (30%, for synthesis), sodium hydroxide pellets and sodium acetate trihydrate were of p.a. grade from Merck (Darmstadt, Germany). Acetonitrile was of HPLC grade from Baker (Deventer, Netherlands), and formic acid (98%, p.a.) and L-phenylalaninol (Pheol) were from Fluka (Buchs, Switzerland); DL-cysteic acid (Cya), β-(2-thienyl)-DL-alanine, (Thia), α-aminoisobutyric acid (Aib), 2-aminoethanol and 4 *M* methanesulfonic acid with 0.2% (w/v) tryptamine as scavenger were purchased from Sigma (St. Louis, MO, USA); L-Cya was from Serva (Heidelberg, Germany), β-casomorphin (bovine) and α_{s1}-exorphin were from Bachem (Bubendorf, Switzerland), and 1 *M* potassium borate buffer was from Pierce (Rockford, IL, USA).

The 6 *M* HCl used for hydrolysis was prepared in the laboratory from concentrated aqueous HCl (p.a., approximately 37%, w/w, Merck) which was diluted with an equal amount of bidistilled water and distilled twice. The bidistilled water exclusively used for all operations was obtained by distillation of reversed-osmosis water (Milli-Q system, Millipore, Bedford, MA, USA) using a quartz still (Model Bi 18 T) from Heraeus (Karlsruhe, Germany). Performic acid for the oxidation of cyst(e)ine containing peptides was freshly prepared by reaction of formic acid (98%) and hydrogen peroxide (30%) (95:5, v/v) for 2 h at ambient temperature [53].

The AA standards were prepared from 100 pmol of L-AAS and 5 pmol of D-AAs which were dissolved in 0.1 *M* HCl (1 l). The unusual amino components (CyA, Aib, Thi, Pheol, aminoethanol) were added, if necessary. If required, an aliquot of a stock solution of the acid-sensitive Trp in 1 *M* $NaHCO_3$ was added to the standard immediately before analysis.

The derivatization reagent (referred to as OPA–IBLC reagent) consisted of 260 m*M* IBLC and 170 m*M* OPA and was dissolved in 1 *M* potassium borate buffer (fluoraldehyde dilutant; Pierce, Rockford, IL, USA; Product No. 27035). The IBLC we used was prepared in our laboratory [46] (99.97 ± 0.01 optical purity) and is obtainable (just as N-isobutyryl-D-cysteine) from Novabiochem-Calbiochem (Läufelfingen, Switzerland) or subsidiaries of Calbiochem (La Jolla, CA, USA); IBLC and IBDC have also been included as Chirabrand reagents in the ChiraSelect series of Fluka.

2.2. Sources of samples

The natural polypeptides bacitracin (ca. 70% Bacitracin A; Product No. B-0125), gramicidin S from *Bacillus brevis* (Product No. G-5127), poly-

Table 1
Quantitative determination (OPA–IBLC) of amino acids in formulations used for parenteral nutrition

Amino acid	Aminosteril				Infesol			
	Declared ($g\,l^{-1}$)	Determined ($g\,l^{-1}$)	S.D. ($n=3$) ($g\,l^{-1}$)	Difference (%)	Declared ($g\,l^{-1}$)	Determined ($g\,l^{-1}$)	S.D. ($n=3$) ($g\,l^{-1}$)	Difference (%)
Asp	–[a]	–	–	–	2.00	2.04	0.02	+2.0
Glu	–	–	–	–	5.00	5.03	0.05	+0.6
Thr	4.21	4.03	0.08	−0.7	1.60	1.59	0.00	−0.6
Gly	15.95	15.49	0.11	−2.9	7.00	7.11	0.10	+1.6
His	2.88	2.82	0.04	−2.1	1.35	1.38	0.04	+2.2
Ala	15.00	14.86	0.03	−0.9	4.00	4.00	0.01	±0.0
Arg	10.64	10.43	0.07	−2.2	4.55	4.38	0.02	−3.7
Val	5.92	5.94	0.02	+0.3	2.25	2.19	0.02	−2.7
Met	4.10	4.09	0.00	−0.2	1.75	1.76	0.02	+0.6
Trp	1.82	1.76	0.03	−3.3	0.50	0.53	0.01	+6.0
Phe	4.82	4.84	0.01	+0.4	3.15	3.19	0.03	+1.3
Ile	4.67	4.63	0.04	−0.9	2.50	2.39	0.00	−4.4
Leu	7.06	6.97	0.02	−1.3	2.75	2.66	0.01	−3.3
Lys	5.97	6.01	0.06	+0.7	2.00	2.18	0.01	+9.0
Pro	15.00	N.D.[a]	–	–	–	–	–	–

[a] N.D. = not determinable; – = not present.

myxin B (Product No. P-1004) and malformin A (Product No. M-2282) were from Sigma; gramicidin A was from Calbiochem, and metanicin C was isolated in our laboratory from *Metarhizium anisopliae* [54]. The synthetic peptide drug D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin was donated by Novabiochem, and the opioidic peptides β-casomorphin and α_{s1}-exorphin were purchased from Bachem and were synthesized by solid-phase peptide synthesis.

The following pharmaceutical AA formulations were investigated (for detailed specifications and additional components we refer to the German Pharmacopoeiae [55,56]).[1]

Aktiferrin capsules (active components in one capsule are 129 mg of DL-Ser and 113.85 mg of $FeSO_4 \cdot 0.8$–$2\ H_2O$), Mg-nor chewing pills (active component in one pill is 1.803 g of magnesium DL-hydrogen aspartate tetrahydrate), Magnesiocard granulate (active component in 5 g of granulate is 1229.6 mg of magnesium L-hydrogen aspartate hydrochloride trihydrate), Lipovitan dragees (active components are 25 mg of DL-Met and 170 mg of cholin hydrogen tartrate in one coated tablet), Tryptocompren tablets (active component is 500 mg of L-Trp in one tablet) were used. The formulations used for parenteral nutrition were "Aminosteril KE 10%", free of carbohydrates, and "Infesol 40", L-AA infusion formulations for parenteral nutrition. For the AA composition we refer to Table 1, for detailed specifications of these pharmaceuticals we refer also to Ref. [55].

2.3. Treatment of samples for analysis

Peptides containing no Cys or Trp (gramicidin S, polymyxin, Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, metanicin C, β-casomorphin, α_{s1}-exorphin) were directly hydrolysed with 6 *M* HCl. Amounts of 0.5 mg of the peptides were placed in Reacti-vials (Wheaton, Milford, NJ, USA), 6 *M* HCl (0.5 ml) was added, the vials were flushed with nitrogen and tightly closed with PTFE-lined screw-caps. Then the vials were

[1] The mention of a product by its trade or proprietary name in this paper may not be considered as a recommendation or otherwise of the particular preparation.

placed in an electrically heated aluminium heating block (Liebisch, Bielefeld, Germany) and hydrolysed for 24 h at 110 ± 1°C. Solvents were removed in a stream of nitrogen, the residues were dissolved in 0.1 *M* HCl (1 ml) and 2-μl aliquots were analyzed. Peptides containing Trp (gramicidin A, malformin A) were hydrolysed with 4 *M* methanesulfonic acid (0.5 ml) for 24 h at 110°C, then water (0.5 ml) was added and 2-μl aliquots were analyzed.

Peptides containing Cys (bradykinin, malformin A) were oxidized with freshly prepared performic acid (see above) prior to total hydrolysis. Amounts of 0.5 mg of the peptides were dissolved in formic acid (1 ml) and methanol (0.5 ml) was added. The solutions were chilled to −10°C and performic acid (2.5 ml) was added. After 2.5 h at −10°C solvents were removed in vacuo using a rotatory evaporator, the residues were dissolved in 6 *M* HCl (0.5 ml) and total hydrolyses using 6 *M* HCl were carried out as described above. After evaporation to dryness, 0.1 *M* HCl (1 ml) was added and 2-μl aliquots were analysed. In the case of malformin A the solution was diluted 1:5 (v/v), in the case of polymyxin and metanicin the solution was diluted 1:10 (v/v) prior to analysis of 2-μl aliquots by HPLC.

For analysing Aktiferrin, one gelatine capsule was dissected and the contents were dissolved in 0.01 *M* HCl (100 ml); an aliquot of the solution was diluted 1:100 (v/v) with 0.01 *M* HCl and 2-μl aliquots were analysed.

For Mg-nor, one chewing pill was dissolved in 0.1 *M* HCl (250 ml), an aliquot was diluted 1:1000 (v/v) with 0.1 *M* HCl and 2-μl aliquots were analysed.

For Lipovitan, one dragee was ground in a mortar, the powder was transferred into a volumetric flask (100 ml), 0.1 *M* HCl (100 ml) was added, the resulting solution was filtered and 2-μl aliquots were analysed.

For Magnesiocard granulate, one package (active component 1229.6 mg of magnesium L-hydrogen aspartate hydrochloride trihydrate in 5 g of granulate) was dissolved in a volumetric flask in bidistilled water (100 ml), the solution was filtered and 2-μl aliquots were analyzed.

For Tryptocompren, 0.1 *M* HCl (100 ml) was added to one tablet in a volumetric flask (100 ml), the resulting solution was filtered, an aliquot was diluted 1:20 (v/v) and 2-μl aliquots were analyzed.

For analysing Aminosteril and Infesol, 1-ml aliquots were diluted 1:2000 and 1:1000 (v/v), respectively, and 2-μl aliquots were analysed.

2.4. Apparatus

The instrument used was an HP 1090 Series L HPLC system comprising a binary DR5 solvent-delivery system, autoinjector–autosampler, electrically heated column compartment and an HP 1046A programmable fluorescence detector with a 280-nm cut-off filter set at an excitation wavelength of 230 nm (band width 25 nm) and an emission wavelength of 445 nm (band width 50 nm). The frequency of the pulsed xenon lamp was 55 Hz, the response time of the detector was set at 500 ms and the photomultiplier tube (PMT) gains at 7–10. Chromatograms were processed with ColorPro Model 7440A plotter and a ThinkJet Model 2225A printer. Operations were controlled by the Pascal ChemStation HP 79994A together with a HP 9000 Series 300 computer, HP 1090 sofrware, floppy disc drive and HP 9153 B hard disc drive.

2.5. Chromatography

The column (250 mm × 4 mm I.D.) was packed with a tested batch of Hypersil ODS, 5 μm (Shandon Scientific, Astmoor, Runcorn, UK) and equipped with a guard column (20 mm × 2.1 mm I.D.) packed with the same stationary phase (columns of this type can be ordered with reference to the applications described in this work from Hewlett-Packard (Waldbronn, Germany) or from the international subsidiaries of HP.

2.6. Derivatization procedure and gradient elution

For derivatization, 5 μl of 0.2 *M* sodium borate buffer of pH 10.4 (Hewlett-Packard, Part

No. 5061-3339), 1 μl of OPA–IBLC reagent (see above) and 2 μl of AA standard (or analyte solution) were drawn up successively by the autosampler and mixed in the derivatization devise of the instrument [46]. The injector was programmed for five mixing cycles in the 8-μl mode (reaction time 2 min); then the mixture was injected onto the column.

For elution a gradient was formed from 23 m*M* sodium acetate adjusted to pH 6.0 (eluent A) and a mixture of methanol–acetonitrile (600:50, v/v) (eluent B). The gradient was formed from 0% B to 53.5% B in 75 min at a flow-rate at 1 ml/min. The temperature of the column was kept at 25°C.

3. Results and discussion

After appropriate treatment for analyses as described in the Experimental section, 2-μl aliquots of filtered samples were automatically derivatized and analyzed by the instrument. Standards were run in parallel and AA stoichiometries of peptides were calculated and normalized with respect to selected AAs. Absolute amounts of pharmaceutical AAs were calculated from the respective peak areas of analytes and standards. Since in the cases of the samples investigated the AA composition was known and no complex matrices (as in the cases of biosamples [48] and foodstuffs [51]) has to be considered, the elegant approach of reversing the elution order of L- and D-AAs by applying the IBDC reagent (for illustrations see Refs. [48]–[50]) was not used.

In the following the analyses are discussed in more detail. The chromatogram of the hydrolysed cyclopeptide antibiotic bacitracin A is shown in Fig. 1 (although actually diastereomers are resolved, for simplicity the names and configurations of the analyte AAs are indicated in all chromatograms). This peptide is a natural mixture of more than nine components with approximately 70% bacitracin A as major compound. The peptide contains a N-terminal thiazoline ring (which might be formally derived from intramolecular condensation of N-terminal Ile and Cys) as well as D-Glu, D-Orn, D-Phe and D-Asp among other L-AAs (cf. structure in Fig. 1). The configurations (D or L) of chiral AAs are indicated, the relative amounts of the major

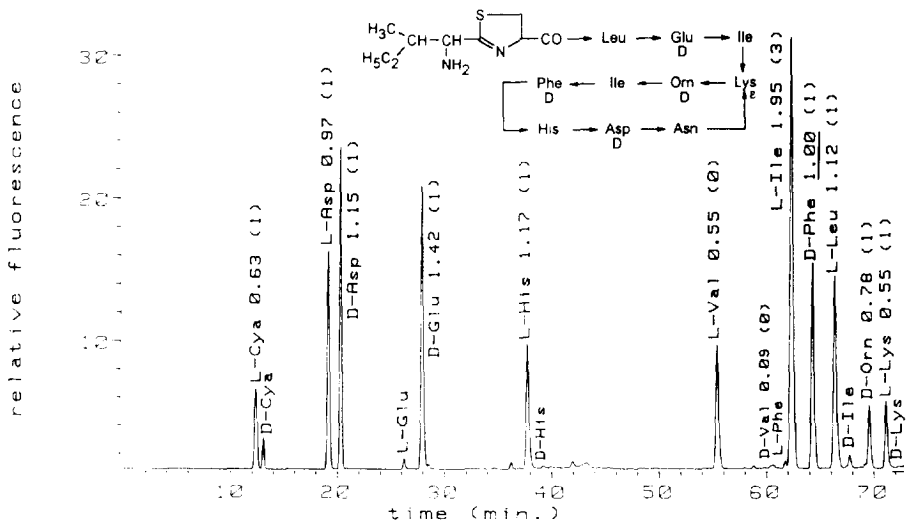

Fig. 1. HPLC of amino acids (AAs) released from bacitracin A after oxidation with performic acid followed by total hydrolysis with 6 *M* HCl (see Experimental); Cya = cysteic acid. Structures of peptides with positions of D-AAs are inserted in all chromatograms; arrows in cyclopeptides indicate peptide linkage from CO → NH bond. AA ratios, normalized with respect to the AA underlined, are displayed. Derivatization of AAs in all chromatograms shown was performed with *o*-phthaldialdehyde–N-isobutyryl-L-cysteine (OPA–IBLC) reagent.

AAs which were found, normalized with respect to D-Phe (underlined), are given and the calculated values are set in parentheses. The peptide was oxidized with performic acid prior to total hydrolysis. As can be seen, the N-terminal thiazoline ring is oxidized and cysteic acid (Cya) is released by total hydrolysis in which D-Cya (21%) is formed. Since free cysteine is known to give a very low fluorescence with OPA and thiols in general [3,48], this oxidative conversion of Cys in Cya is suitable for the determination of amounts and configuration of cysteine and cystine (see also malformin A, Fig. 2). Taking into account that the peptide contains approximately 70% of peptide component A, the found and calculated AA ratios (based on Phe) are in acceptable agreement with the structure. As can be seen nicely with D-Asp and L-Asn (the latter is hydrolysed to L-Asp), the method allows the simultaneous quantification of both enantiomers. The configuration of D-Glu, D-Orn and D-Phe in the peptide is also confirmed by the method. Further, the formation of low amounts of the opposite enantiomer of those AA (either D- or L) forming the major part of the peptide is detectable in the total hydrolysate. Thus L-Glu (3.7%), D-His (1.8%), L-Phe (2.9%), D-Ile (3.2%) and D-Lys (1.4%) are determined. This is attributed to the well known, acid-catalysed background racemization of AAs in peptides or proteins [57]. Interestingly, a relative high amount of L-Val is released from the peptide which indicates, together with the non-stoichiometry of D-Glu and L-Lys, the occurrence of further peptides in the sample.

The cyclopeptide toxin malformin A (Fig. 2) contains two peptide linked and disulfide-bridged D-cysteines, as well as D-Leu, L-Ile and L-Val. Performic acid oxidation and total hydrolysis releases D-Cya, whereby, in analogy to bacitracin, L-Cya (11.5%) is formed. Low amounts of D-Val (1.7%), D-Leu (4.3%) and D-Ile (1.6%) are formed during hydrolysis.

The linear peptide antibiotic gramicidin A is N-formylated and contains C-terminal peptide bonded aminoethanol (Fig. 3). Furthermore, of the four Val, two have the D- and two the L-configuration. The two L-Trp would be destroyed under the conditions of standard hydrolysis using 6 *M* HCl at 110°C for 24 h. However, replacement of 6 *M* HCl by 4 *M* methanesulfonic acid, with addition of tryptamine as scavenger has been reported to give good recoveries of Trp [3,58]. This is confirmed by the data shown in Fig. 3 which also verifys the assignment of the configuration of AAs in gramicidin A. Again, low amounts of D-Ala (2.7%), D-Trp (2.3%) and D-Leu (3.8%) formed from the opposite enantiomers in the course of hydrolysis.

The chromatogram of a total hydrolysate (6 *M*

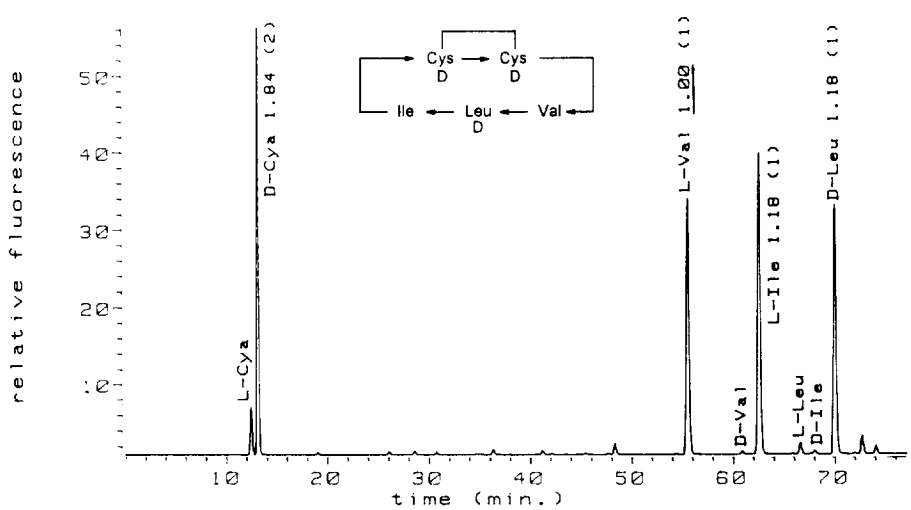

Fig. 2. HPLC of AAs released from malformin A after oxidation with performic acid followed by total hydrolysis with 6 *M* HCl.

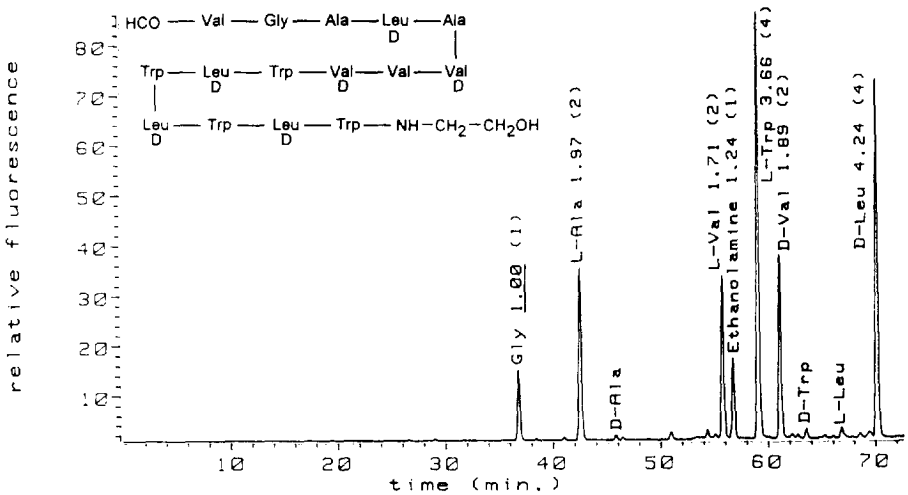

Fig. 3. HPLC of AAs released from gramicidin A hydrolysed with 4 *M* methanesulfonic acid (see Experimental); HCO = formyl, C-terminal amino alcohol = ethanolamine.

HCl) of the cyclopeptide antibiotic gramicidin S is shown in Fig. 4. The presence of D-Phe is confirmed and low amounts of L-Phe (1.6%) and D-Val (0.7%) are determinable. As can be seen, low amounts of D-Orn and D-Leu, arising from the corresponding L-enantiomers, are eluting together. The amounts of L-Pro are not determinable owing to the inability of secondary amines to react with OPA–thiol reagents.

The chromatogram of a total hydrolysate (6 *M* HCl) of the cyclopeptide antibiotic polymyxin B is shown in Fig. 5. The N-terminus of this decapeptide is blocked by a 6-methyloctanoyl (6-MeOct) residue and the peptide contains one of each of L-Thr, L-Leu and D-Phe as well as six L-2,4-diamino-*n*-butyric acid (Dba) moieties. Dba is not determinable under the standard conditions of the chromatogram; the other AAs yield satisfactory stoichiometries. Low amounts of L-Phe (1.4%) and D-Leu (2.0%) are formed during hydrolysis.

The peptaibol antibiotic metanicin C [54] (Fig. 6) contains nine residues of the non-protein, achiral α-aminoisobutyric acid (2-methylalanine,

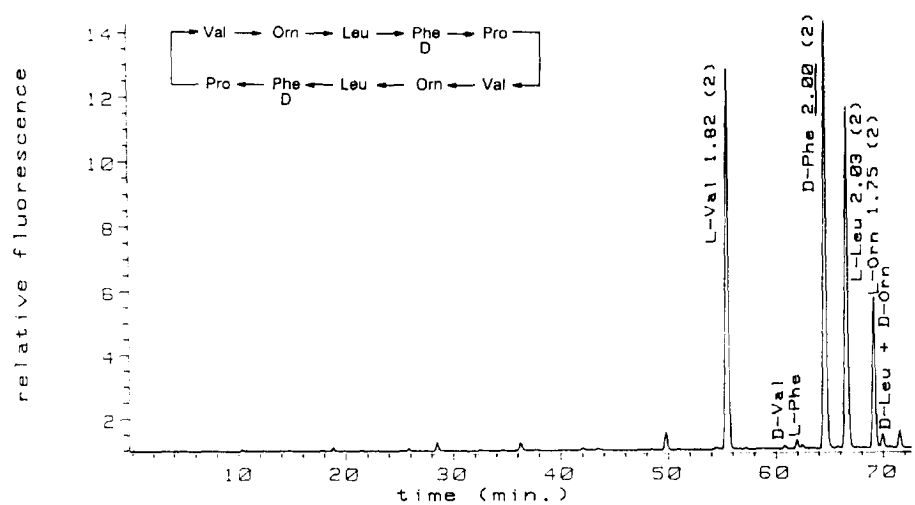

Fig. 4. HPLC of AAs released from gramicidin S, hydrolyzed with 6 *M* HCl.

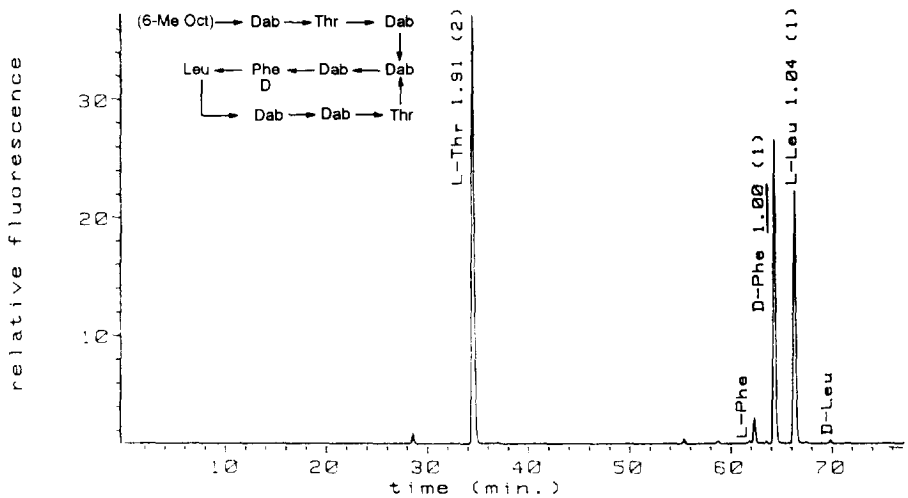

Fig. 5. HPLC of AAs released from polymycin B, hydrolyzed with 6 *M* HCl; 6-MeOct = 6-methyloctanoyl; Dab = L-2,4-diaminobutyric acid.

Aib). The N-terminus is acetylated and the C-terminus blocked by the peptide-bonded L-phenylalaninol (Pheol). The stoichiometry of the amino components is very satisfactory also with respect to Aib, which gives, owing to steric hindrance of the *gem* dimethyl groups, a much lower derivatization yield in general in comparison to other α-AAS [59]. L-Pheol, which is, as a result of its basicity, not eluted from the column under the conditions of traditional AA analysis by ion-exchange chromatography, is determinable by the method. The fluorescence detector response of Aib, in comparison to Leu, is approximately 50% in metanicin. D-Pheol, if present, would not be separated from L-Pheol under the chromatographic conditions described (the configuration of L-Pheol in metanicin was determined by chiral gas chromatography on

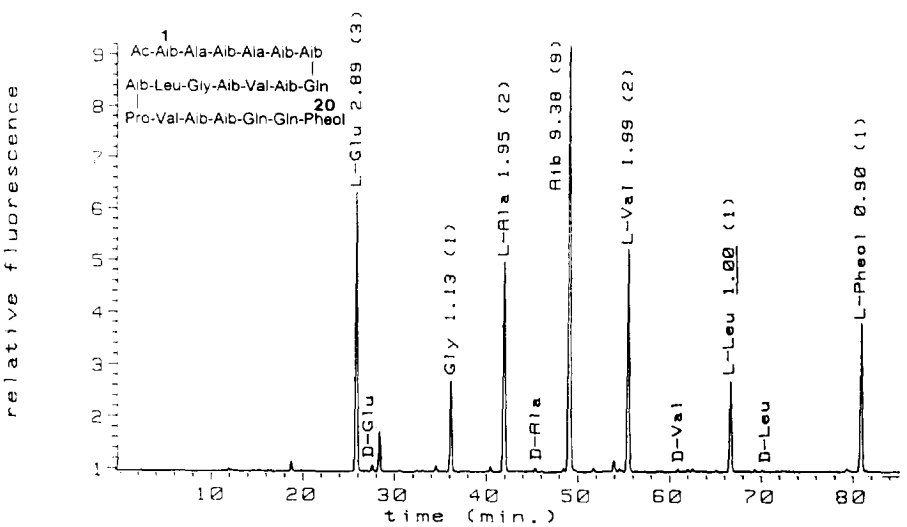

Fig. 6. HPLC of AAs released from metanicin C, hydrolyzed with 6 *M* HCl; Ac = acetyl, Aib = α-aminoisobutyric acid (2-methylalanine); Pheol = L-phenylalaninol.

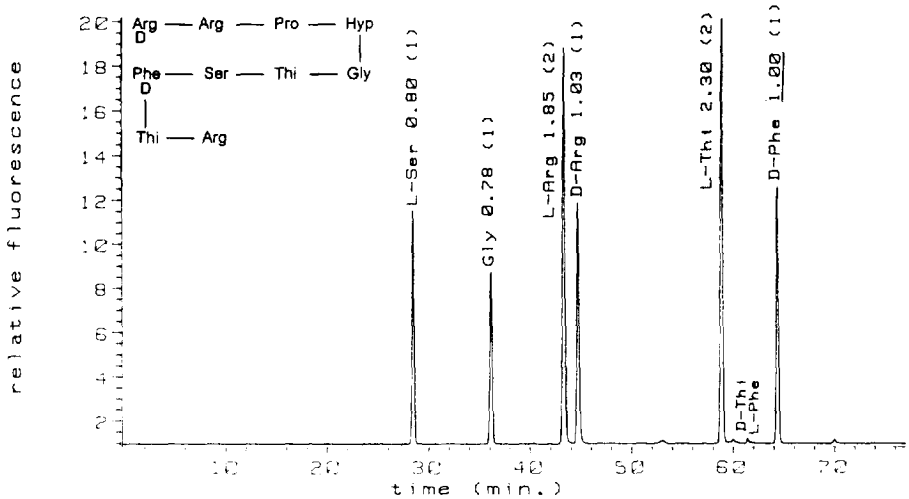

Fig. 7. HPLC of AAs released from Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, hydrolyzed with 6 *M* HCl.

Chirasil-L-Val [57]). Low amounts of D-Glu (2.5%), D-Ala (2.25%), D-Val (1.0%) and D-Leu (1.85%) are formed during hydrolysis.

The chromatogram of the hydrolysate (6 *M* HCl) of the bradykinin analog peptide drug D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin is shown in Fig. 7. The expected D-AAs are found and the AA stoichiometry of the peptide is satisfactory also for the unusual 2-(thienyl)-L-alanine (Thi). Amounts of D-Thi (1.2%) and L-Phe (1.0%) are detectable, whereas L-Hyp, as secondary AA, is not determinable.

The chromatograms of hydrolysates (6 *M* HCl) of the opioid peptides β-casomorphin and α_{s1}-exorphin are shown in Figs. 8 and 9, respectively. These peptides contain L-AA exclusively and the AA stoichiometries are in agreement with the compositions of the peptides. Since these peptides had been synthesized by solid-phase procedures [60], the integrity of the chi-

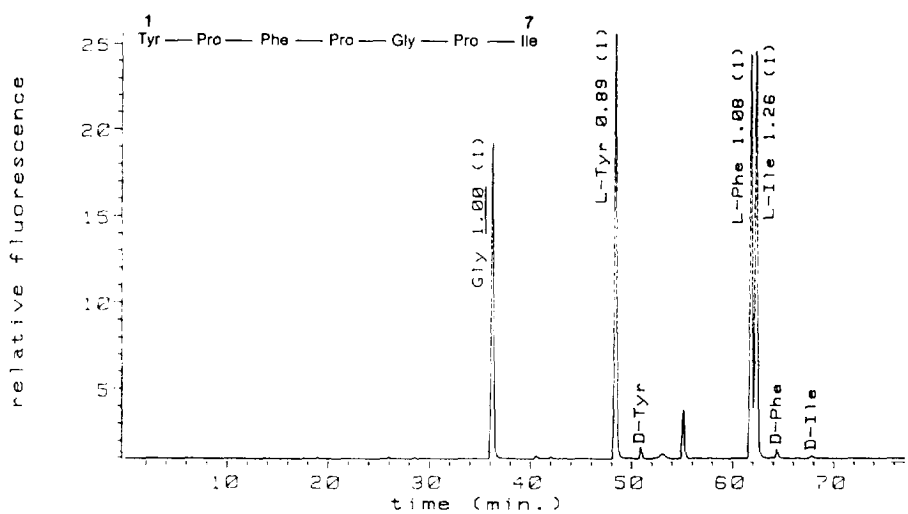

Fig. 8. HPLC of AAs released from β-casomorphin, hydrolyzed with 6 *M* HCl.

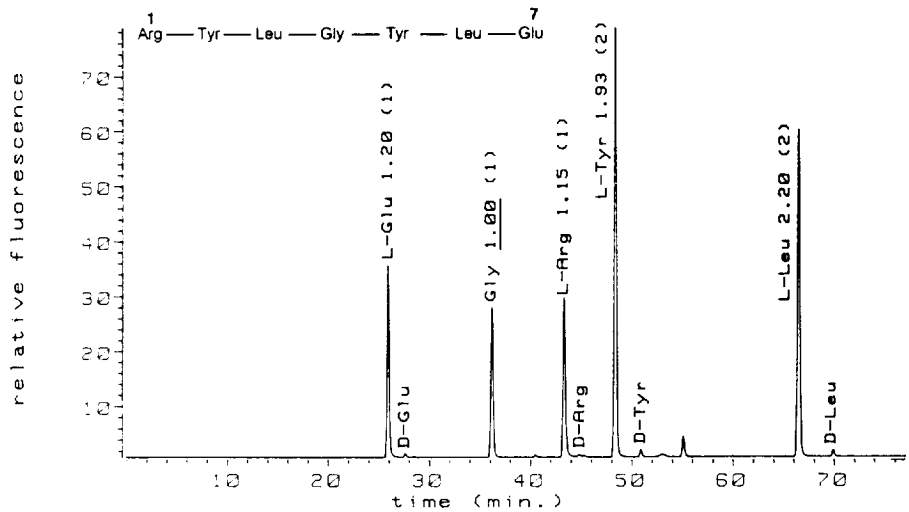

Fig. 9. HPLC of AAs released from α_{s1}-exorphin, hydrolyzed with 6 *M* HCl.

rality of the respective C-terminal AAs, viz. L-Ile and L-Glu, was of particular interest. Analysis gave D-Ile (0.8%), D-Tyr (2.6%) and L-Phe (2.1%) for β-casomorphin, and D-Glu (2.1%), D-Leu (2.1%), D-Arg (1.7%), D-Tyr (1.7%) and D-Leu (2.1%) for α_{s1}-exorphin. From the data it is evident that no noticeable racemization occurred in the course of anchoring the C-terminal AAs on the solid support or sequence elongations during solid-phase syntheses [60].

Sections of the chromatograms of the analyses of the DL-AA drugs Aktiferrin (Fe^{3+} complex with DL-Ser, used in the therapy for hypoferric anemia as well as prelateral and lateral iron deficiencies [61]), Mg-nor (Mg salt of DL-Asp, used in the treatment of magnesium deficiency

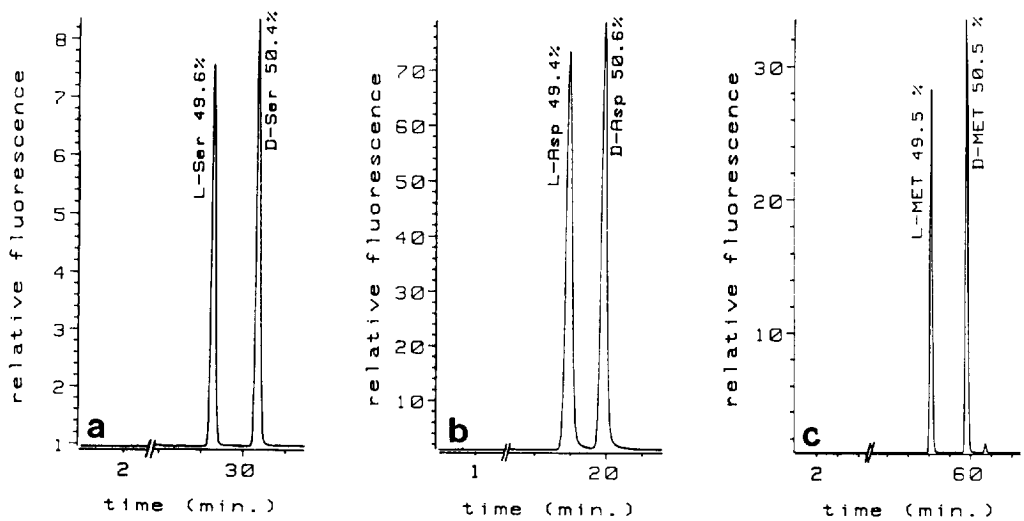

Fig. 10. Elution profiles and ratios of D- and L-enantiomers in the racemic AA drugs (a) Aktiferrin (DL-Ser), (b) Mg-nor (DL-Asp) and (c) Lipovitan (DL-Met); racemic AAs in parentheses, for other components of drugs see Experimental.

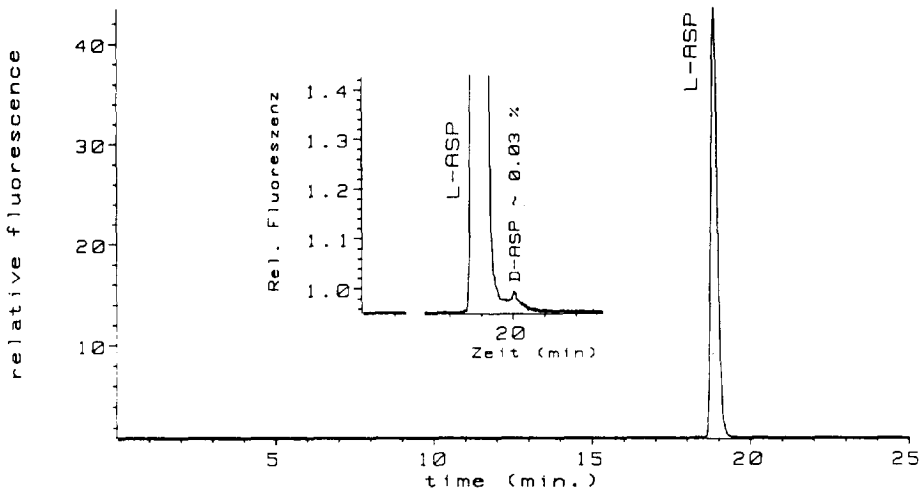

Fig. 11. Chromatogram and expanded plot of L-Asp determined in Magnesiocard.

arising from dysfunctions of muscular activity) and Lipovitan (DL-Met and vitamins, used as a liver remedy and for vitamin substitution) are shown in Fig. 10a–c, respectively. The analyses confirm that the AAs in these drugs are indeed (almost) racemic and that the drugs, after being dissolved and filtered, if necessary, can be investigated directly by the method described.

The chromatogram and an expanded plot of the Magnesiocard (Mg^{2+} salt of L-Asp, used as a prophylaxis and treatment for cardiac infarction) is shown in Fig. 11. The analysis demonstrates the very high optical purity of the drug. The apparent traces of ca. 0.03% D-Asp which are detectable are attributed to the diastereomer formed from trace amounts of N-isobutyryl-D-Cys which are present in the IBLC reagent (see Experimental). An amount of 4.8 mmol of L-Asp in 5 g of the drug was determined and agrees satisfactorily with 5.0 mmol of L-Asp declared for the drug.

The chromatogram and expanded plot of the L-Trp drug Tryptocompren (antidepressant and sedativum) are presented in Fig. 12. No D-Trp

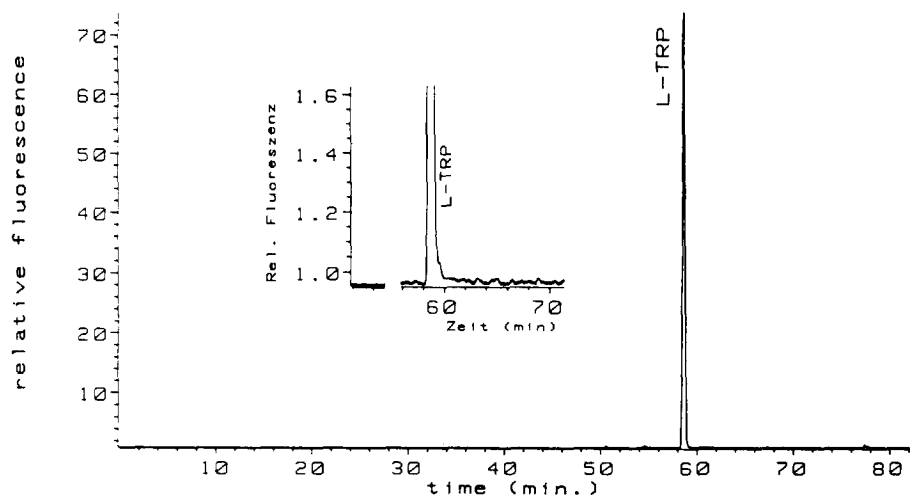

Fig. 12. Chromatogram and expanded plot of L-Trp determined in Tryptocompren.

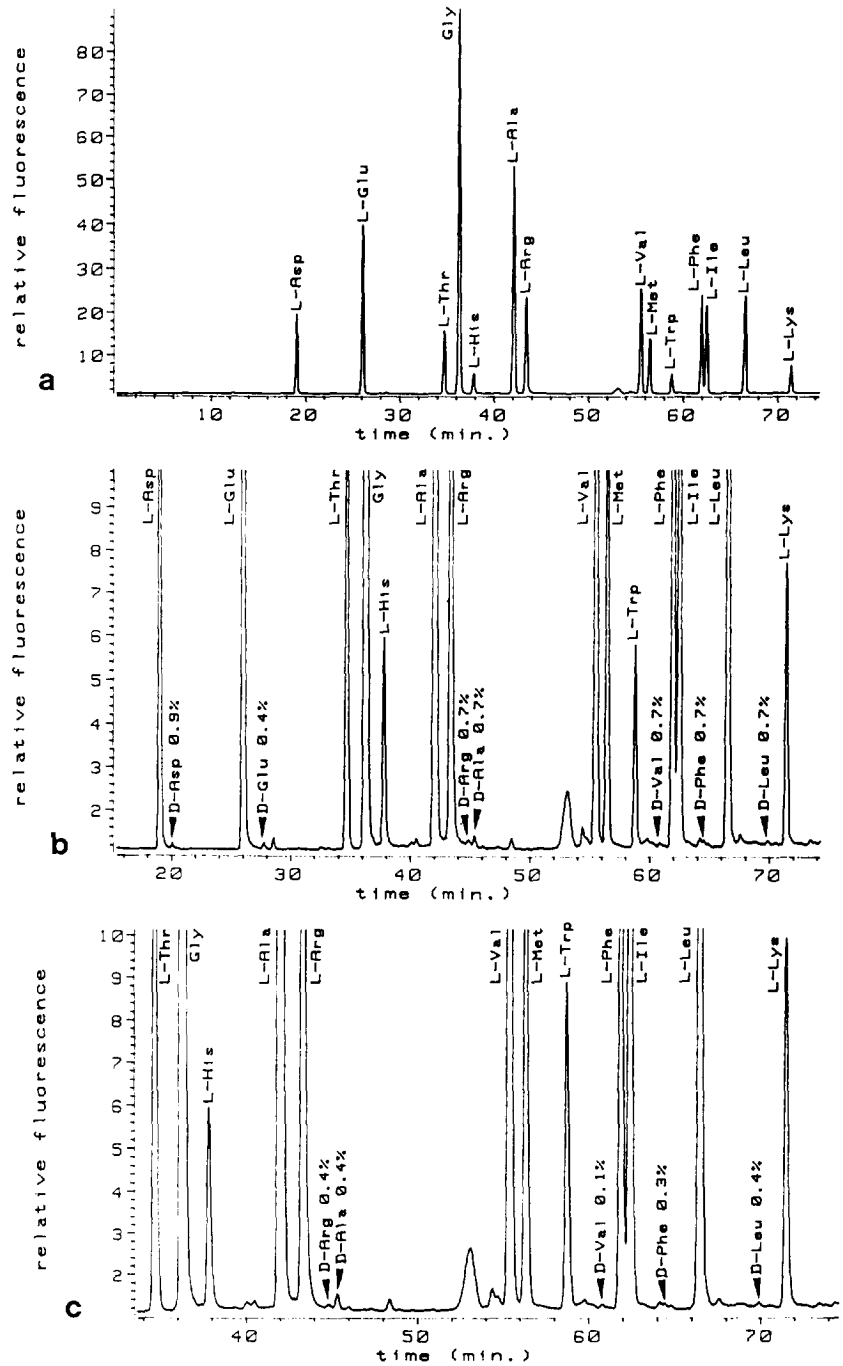

Fig. 13. Elution profiles of AAs determined in formulations used for intravenous administration in parenteral nutrition. (a) HPLC of Infesol and (b) expanded plot of this chromatogram; (c) expanded plot of Aminosteril. For composition see Experimental and for quantification of AAs see Table 1.

(which would elute at 63 min) is detectable in the expanded plot. The nature of the shoulder of the L-Trp, which is only recognized in the zoomed chromatogram, is not known.

Chromatograms of the AA-containing formulation Infesol 40 (indicated for mediating or eliminating protein deficiency states) are shown in Fig. 13a and b, and the elution profile of AA of Aminosteril KE 10% (employed for partial parenteral feeding) is shown in Fig. 13c. The declared and quantitatively determined amounts of α-AAs listed in Table 1 (see Experimental) agree favorably. In both solutions very low amounts of certain D-AAs could be detected in expanded plots of the chromatograms; for Infesol 40 this is shown in Fig. 13b and for Aminosteril in Fig. 13c. In Infesol, D-Asp (0.9%), D-Glu (0.4%), D-Ala (0.7%), D-Arg (0.7%), D-Val (0.7%), D-Phe (0.7%) and D-Leu (0.8%) were detectable, and in Aminosteril, low amounts of D-Arg (0.4%), D-Ala (0.4%), D-Val (0.1%), D-Phe (0.3%) and D-Leu (0.4%) were determined. Other D-AAs (if present) are <0.1% in both formulations. Thus, the sum of determinable D-AAs amounts to 128 mg l^{-1} in Infesol and to 149 mg l^{-1} in Aminosteril. These analyses demonstrate the suitability of the liquid chromatographic method for the simultaneous quantification of D- and L-AAs in these formulations. It is worth noting that the analyses are not perturbed by the presence of other components such as electrolytes (present in both samples), malic acid (8.08 g l^{-1} in Aminosteril) or sorbitol (100 g l^{-1} in Infesol) [55]. For a discussion of the physiological relevance of D-AAs we refer to previous publications [48,62,63] and the references cited therein.

References

[1] M. Roth, Anal. Chem., 43 (1971) 880.
[2] D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, Anal. Chem., 51 (1979) 1338.
[3] H. Umgat, P. Kucera and L.-F. Wen, J. Chromatogr., 239 (1982) 463.
[4] H.W. Jarrett, K.D. Cooksy, B. Ellis and J.M. Anderson, Anal. Biochem., 153 (1986) 189.
[5] P. Fürst, L. Pollack, T.A. Graser, H. Godel and P. Stehle, J. Chromatogr., 499 (1990) 557.
[6] G. Georgi, C. Pietsch and G. Sawatzki, J. Chromatogr., 613 (1993) 35.
[7] M.C. Roach and M.D. Harmony, Anal. Chem., 59 (1987) 411.
[8] S.S. Simons and D.F. Johnson, J. Am. Chem. Soc., 98 (1976) 7098.
[9] S.S. Simons and D.F. Johnson, J. Org. Chem., 43 (1978) 2886.
[10] O.S. Wong, L.A. Sternson and R.L. Schowen, J. Am. Chem. Soc., 107 (1985) 6421.
[11] R. Schuster, J. Chromatogr., 431 (1988) 271.
[12] D.T. Blankenship, M.A. Krivanek, B.L. Ackermann and A.D. Cardin, Anal. Biochem., 178 (1989) 227.
[13] V. Barkholt and A.L. Jensen, Anal. Biochem., 177 (1989) 318.
[14] P. Seitz and H. Godel, Biotech. Forum Eur., 9 (1992) 710.
[15] D.W. Aswad, Anal. Biochem., 137 (1984) 405.
[16] J.J. Usher, M. Lewis and D.W. Hughes, Anal. Biochem., 1490 (1985) 105.
[17] R.H. Buck and K. Krummen, J. Chromatogr., 315 (1984) 279.
[18] N. Nimura and T. Kinoshita, J. Chromatogr., 352 (1986) 169.
[19] M. Fujiwara, Y. Ishida, N. Nimura, A. Toyama and T. Kinoshita, Anal. Biochem., 166 (1987) 72.
[20] R.H. Buck and K. Krummen, J. Chromatogr., 387 (1987) 255.
[21] T. Takeuchi, T. Niwa and D. Ishii, J. High Resol. Chromatogr. Chromatogr. Commun., 11 (1988) 343.
[22] M.R. Euerby, L.Z. Partridge and P. Rajani, J. Chromatogr., 447 (1988) 392.
[23] J. Florance, A. Galdes, Z. Konteatis, Z. Kosarych, K. Langer and C. Martucci, J. Chromatogr., 414 (1987) 313.
[24] M. Maurs, F. Trigalo and R. Azerad, J. Chromatogr., 440 (1988) 209.
[25] A. Duchateau, M. Crombach, J. Kamphuis, W.H.J. Boesten, H.E. Schoemaker and E.M. Meijer, J. Chromatogr., 471 (1989) 263.
[26] H. Brückner and S. Zivny, Amino Acids, 4 (1993) 157.
[27] M. Zhao and J.L. Bada, J. Chromatogr., 690 (1995) 55.
[28] G. Flesch and P. Degen, in 15th International Symposium on Column Liquid Chromatography, Basel, June 3–7, 1991, Abstract P32/2.
[29] M.R. Euerby, L.Z. Partridge and P.B. Nunn, J. Chromatogr., 469 (1989) 412.
[30] M.R. Euerby, P.B. Nunn and L.Z. Partridge, J. Chromatogr., 466 (1989) 407.
[31] S. Einarsson, S. Folestad and B. Josefsson, J. Liq. Chromatogr., 10 (1987) 1589.
[32] A. Jegorov, J. Tříska, T. Trnka and M. Černý, J. Chromatogr., 434 (1988) 417.
[33] A. Jegorov, V. Maťha, T. Trnka and M. Černý, J. High Resolut. Chromatogr., 13 (1990) 718.

[34] A. Jegorov, J. Tříska and T. Trnka, J. Chromatogr., 673 (1994) 286.
[35] D.M. Desai and J. Gal, J. Chromatogr., 629 (1993) 215.
[36] H. Goetz and B. Glatz, Application Note, Publication No. 12-5952-0029 (10/89), Hewlett-Packard, 1989.
[37] G. Höfle and N. Bedorf, in Wissenschaftliche Ergebnissberichte der Gesellschaft für Biotechnologische Forschung, Gesellschaft für Biotechnologische Forschung, Braunschweig, 1987, p. 90.
[38] H. Brückner, R. Wittner and H. Godel, in G. Lubec and G.A. Rosenthal (Editors), Amino Acids: Chemistry, Biology and Medicine; Proceedings of the 1st International Congress Therapy with Amino Acids and Analogs, Vienna, August 7–12, 1989, Escom Science Publishers, Leiden, 1990, pp. 143–151.
[39] A.L.L. Duchateau, H. Knuts, J.M.M. Boesten and J.J. Guns, J. Chromatogr., 623 (1992) 237.
[40] L. Kang and R.H. Buck, Amino Acids, 2 (1992) 103.
[41] R.J.H. Houben, H. Gielen and S. van der Waals, J. Chromatogr., 634 (1993) 317.
[42] C. Dette and H. Wätzig, Electrophoresis, 15 (1994) 763.
[43] H. Brückner, R. Wittner and H. Godel, in 8th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Copenhagen, October 31–November 2, 1988, Abstract 205, p. 19.
[44] H. Brückner, R. Wittner and H. Godel, J. Chromatogr., 476 (1989) 73.
[45] M.R. Euerby, L.Z. Partridge and W.A. Gibbons, J. Chromatogr., 483 (1989) 239.
[46] H. Brückner, R. Wittner and H. Godel, Chromatographia, 32 (1991) 383.
[47] H. Brückner, Ch. Keller-Hoehl and R. Wittner, in D. Brandenburg, V. Ivanov and W. Voelter (Editors), Chemistry of Peptides and Proteins, Vols. 5–6, Proceedings of the 7th USSR–FRG Symposium on Chemistry of Peptides and Proteins, Dilizhan, September 23–30, 1989, and Proceedings of the 8th FRG–USSR Symposium on Chemistry of Peptides and Proteins, Aachen, September 29–October 3, 1991, DWI Reports, Deutsches Wollforschungsinstitut an der TH Aachen e.V., Vol. 112A–B, Part A, Verlag Mainz, Aachen, 1993, pp. 145–156.
[48] H. Brückner, S. Haasmann, M. Langer, T. Westhauser, R. Wittner and H. Godel, J. Chromatogr., 666 (1994) 259.
[49] H. Brückner, P. Jaek, M. Langer and H. Godel, Amino Acids, 2 (1992) 271.
[50] H. Brückner and T. Westhauser, Chromatographia, 39 (1994) 419.
[51] H. Brückner, M. Langer, T. Westhauser and H. Godel, J. Chromatogr., 697 (1995) 229.
[52] H. Brückner, M. Langer and H. Godel, in R.H. Angeletti (Editor), Techniques in Protein Chemistry III, Academic Press, San Diego, CA, 1992, pp. 243–247.
[53] C.H.W. Hirs, Methods Enzymol., 11 (1967) 197.
[54] H. Brückner and A. Kimonyo, in H.L.S. Maia (Editor), Peptides 1994, Proceedings of the 23rd European Peptide Symposium, Braga, September 4–10, 1994, Escom Science Publishers, Leiden, 1995, pp. 429–430.
[55] Bundesverband der Pharmazeutischen Industrie (Ed.), Rote Liste 1994, Arzneimittelverzeichnis des BPI, Editio Cantor, Aulendorf/Württemberg, 1994.
[56] Institut für Arzneimittelwesen der DDR (Ed.), Arzneimittelverzeichnis 1988, Teil I, 22. Auflage, VEB Verlag Volk und Gesundheit, Berlin, 1989.
[57] H. Frank, W. Woiwode, G. Nicholson and E. Bayer, Liebigs Ann. Chem., (1981) 354.
[58] R.J. Simpson, M.R. Neuberger and T.Y. Yu, J. Biol. Chem., 2512 (1976) 1936.
[59] H. Brückner, I. Bosch, T. Graser and P. Fürst, J. Chromatogr., 386 (1987) 251.
[60] J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, Pierce, Rockford, IL, 1984.
[61] D. Kraft and P. Koeppe, Arzneim. Forsch. (Drug Res.), 25 (1975) 1319.
[62] H. Brückner and M. Hausch, J. Chromatogr., 614 (1993) 7.
[63] H. Brückner, D. Becker and M. Lüpke, Chirality, 5 (1993) 385.