ELSEVIER

Journal of Chromatography B, 728 (1999) 157–166

JOURNAL OF CHROMATOGRAPHY B

Quantitative determination of free intracellular amino acids in single human polymorphonuclear leucocytes Recent developments in sample preparation and high-performance liquid chromatography

J. Mühling[a,*], M. Fuchs[b], M.G. Dehne[a], A. Sablotzki[a], T. Menges[a], B. Weber[a], G. Hempelmann[a]

[a]*Department of Anaesthesiology and Intensive Care Medicine, Justus-Liebig-University, Rudolf-Buchheim-Strasse 7, 35385 Giessen, Germany*

[b]*Firma Dr. Ing. Herbert Knauer GmbH, Berlin, Germany*

Received 24 December 1998; received in revised form 13 March 1999; accepted 15 March 1999

Abstract

The described procedure allows quantitative, highly precise and reproducible analysis of free amino acid concentrations in single polymorphonuclear leucocytes (PMLs). This method is superior to previously described procedures with regard to sample size, PML separation, sample preparation and stability, as well as the chosen fluorescence high-performance liquid chromatography procedure, and can satisfy the high demands for ultra-sensitive and comprehensive amino acid analysis, especially for the continuous surveillance of severe diseases and organ dysfunction.

Keywords: Polymorphonuclear leucocytes; Amino acids

1. Introduction

The highly specialized measurement of free amino acids has increased significantly in importance over the last few years, both for the comprehensive analysis of metabolic and nutritional issues as well as for investigation of severe disorders or complex pathophysiological functional disorders of vitally important organ systems. In this respect, the monitoring of intracellular amino acids is of particular interest since physiological cell metabolism and basic cell function relies upon a balanced intracellular amino acid content and the cell membrane-mediated separation of cellular amino acids from the extracellular plasma amino acid pool. However, tissue biopsies to obtain amino acids from liver, kidney or muscle cells (supplementary to routine laboratory procedures that are already available [1,2]), which should optimally be performed at least once daily for continuous monitoring, are not advisable with critical disease courses, not only for methodological reasons, but also on ethical grounds. Rapidly dividing cells that have high metabolic

*Corresponding author. Tel.: +49-0641-99-44401; fax: +49-0641-99-44409.

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00114-0

activity and that are important under both anabolic and catabolic pathophysiological conditions are particularly suited for complex investigations of amino acid metabolism. In more recent studies, attempts have therefore been made to measure the distribution of intracellular amino acids in human polymorphonuclear leucocytes (PMLs). In particular, PMLs are important components of the immunological defense system, which protects humans from invading-bacteria. The importance of their role becomes obvious especially when their numbers are reduced or their functions are impaired [3]. Using PMLs has the clear advantage that the sampling of circulating PMLs in peripheral blood is easy and, most importantly, can be performed in a non-invasive fashion. Since they are similar to other nucleated and differentiated tissue cells with regard to their major metabolic pathways, they appear to be a particularly suitable cell model for performing biochemical and physiological studies [4–7].

The methodological requirements for continuous monitoring of free intracellular amino acids in PMLs are very high, especially with regard to the monitoring of diseases and organ dysfunction that requires intensive care. With many procedures, it is not possible to isolate 'metabolism-neutral' PMLs in a rapid way that does not damage them. Such a procedure must preserve the status quo of the amino acid content during blood sampling and must result in the retention of full cellular viability (without prematurely destroying cellular integrity during the purification procedure) and a high state of purity. Another significant problem is the lack of comparability between previously published experimental findings. This is due, on the one hand, to the variety of techniques employed for cell separation and, on the other hand (and more significantly), to the variety of ways in which the intracellular amino acid concentrations are described [8−15]. Relating the concentrations to a biological parameter which can itself be changed during the course of severe diseases, or that can be subject to acute changes in other disease courses, is, in our opinion, fraught with potential problems. Another problem is that many methods are inadequate regarding the procedures for sample preparation (cytolysis, protection from hydrolysis, amino acid extraction) and maintenance of sample stability [long-term storage and stability before analysis by high-performance liquid chromatography (HPLC)]. The choice of fluorescence HPLC procedure is also of great importance in achieving appropriate and exact amino acid analysis. The goal of this study was therefore to develop a comprehensive and highly precise procedure for analyzing amino acids in PMLs that fulfilled the demands for continuous surveillance in the most severe forms of disease.

2. Experimental

This study was approved by the local ethics committee of the Justus-Liebig-University of Giessen. Eighty-five volunteers (46 male, 39 female), aged between 23 and 35 years (mean, 28 years) with an average height of 174 cm (range, 166–192 cm) and weight of 69 kg (range, 55–98 kg), were selected and those volunteers with metabolic (diabetes, etc.), cardiopulmonary, neurological or allergic diseases as well as those taking any drugs were excluded from the study. Whole blood samples (10 ml) were taken from each volunteer (following 12 h of sobriety) between 9 and 10 a.m. (drawn into lithium heparinate plastic tubes), to exclude potential circadian variations, and were cooled immediately in an iced water bath at 4°C before further processing. Phenyl methyl sulfonyl fluoride (PMSF; 100 μg/ml), 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml antipain (all from Sigma, St. Louis, MO, USA) were added to each plastic heparin tube before the blood samples were added, to inhibit proteases within the blood samples.

2.1. Highly selective separation of PMLs from whole blood

Extraction of PMLs was accomplished using a cooled (4°C) Percoll® gradient (Pharmacia, Uppsala, Sweden). Volumes (4 ml) of the cooled and heparinized whole blood samples were overlaid into a previously prepared and precooled (4°C) 70–55% (in 0.9% NaCl) Percoll® gradient before centrifugation at 350 *g* for 15 min at 4°C (Biofuge®, Heraeus, Hanau, Germany). After separation, the PMLs (which were present as a small layer between the

erythrocyte and monocyte layers) were carefully removed from the sample and suspended in 10 ml of cooled (4°C) PBS® stock buffer that had been diluted 1:10 (v/v) (10× PBS® stock buffer, without $Ca^{2+}Ca/Mg^{2+}$; Gibco BRL, UK). After a second centrifugation step (350 *g* for 5 min at 4°C), the PBS buffer was discarded and the erythrocytes remaining in the sample were hypotonically lysed using 2 ml of cooled (4°C) distilled water (Pharmacia) [12]. After 20 s, the PML samples were brought back to isotonicity (by the addition of 1 ml of 2.7% NaCl at 4°C; Merck, Darmstadt, Germany) and were resuspended by adding 10 ml of diluted stock PBS buffer. After a third centrifugation step (350 *g* for 5 min at 4°C), the PBS buffer was again discarded and two aliquots of resuspended sample were removed. Overall, cell fractionation lasted for 34±4 min on average. Immediately after withdrawal and preparation, the extracted and cooled PML samples were frozen at −80°C before lyophilization (freeze-drying under a temperature at −80°C, CIT-2®, Heraeus, Germany). This allowed a metabolically preserving and non-chemically mediated PML lysis as well as a long stability during storage. Samples prepared in this manner were stored at −80°C until further analysis, i.e., a period not exceeding six months. Both the purity (determined in duplicate; dying using türks solution®, Merck) and vitality (exclusion of trypan-blue®, Merck) of the PML samples were verified subsequently by light microscopy (Zeiss, Oberkochen, Germany) [16]. Samples with a PML purity <96% and those with more than 4% vitality were discarded.

2.2. Sample preparation

As is apparent from the pipetting scheme illustrated in Fig. 1, the lyophilized samples were solubilized in an 80% methanol–water mixture (80:20, v/v) (Mallinckrodt Baker, Deventer, The Netherlands) to guarantee the short-term stability before the column-derivitization procedure (chemical preservation). This standard extraction buffer also contained the amino acid L-homoserine as an internal standard for the HPLC procedure [17]; L-homoserine is an amino acid that does not exist in the body under physiological or pathophysiological conditions.

2.3. Automated precolumn derivitization

As is apparent from the pipetting scheme illustrated in Fig. 1, methanolic extracts of the various lyophilized samples were prepared by repeated manual pipetting using a standard extraction buffer before the start of the precolumn derivitization step. After 5-min incubation and 3 min of centrifugation (3000 *g*, Rotixa/KS®, Tuttlingen, Germany), samples (300 μl of the extract) were transferred to a special sample tube (2-CRV®, Chromacoll, Trumbull, USA) where 120 μl of alkaline 0.5 *M* borate buffer (Merck) and 60 μl of *o*-phthaldialdehyde–2-mercaptoethanol (OPA; Merck) were added automatically. This base derivitization was stopped after exactly 120 s by neutralization with 30 μl of 0.75 *M* HCl (Merck). The mixture was then transferred to a rarefaction vial and diluted 2:3 with eluent A (see below), and 25 μl of this mixture was injected into the column.

2.4. Matrix-dependent calibration

According to the results of Fuchs [18,19], standard samples (including all of the amino acids that we measured in PMLs) have been prepared in our laboratory. For this purpose, analytically pure amino acids (Sigma) were dissolved in pure physiological saline. Human serum albumin (4%; Merck) was also mixed with the standard samples. The standard samples prepared in this manner were stored immediately at −80°C and lyophilized.

2.5. Equipment

The fluorescence HPLC system consisted of a hydrostatic gradient pump, a controller for gradient programming (600 E®, Waters, USA) and a programmable autosampler for the automated derivatization procedures (Triathlon®, Spark, The Netherlands) within a rheodyne injection valve and a 100-μl filling loop (AS 300®, Sunchrom, Friedrichsdorf, Germany). The following column was used for separation: Nova-Pak®, 300×3.9 mm I.D.; RP-C-18, 60 Å, 4 μm (Waters, USA). Column temperature was maintained at 35°C using a column oven (Knauer, Berlin, Germany). Fluorescence was routinely monitored using a fluorescence spectrome-

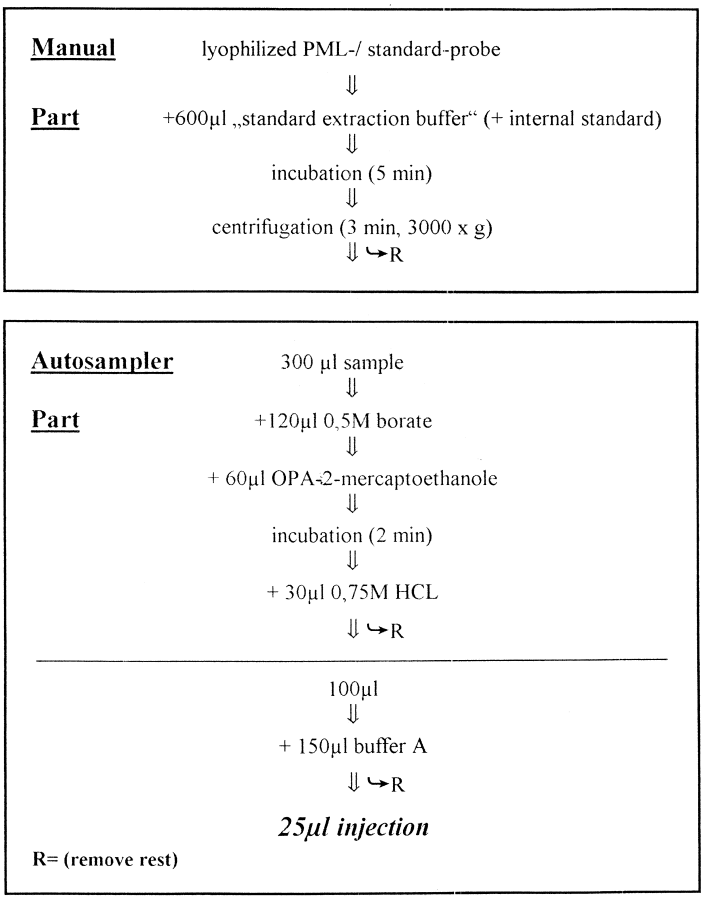

Fig. 1. Derivatization procedure with *o*-phthaldialdehyde–mercaptoethanol for amino acid standards and measurement of free amino acids in PMLs, respectively. Manual and automatic (precolumn derivatization) parts of the preparation process are shown (for details, see Experimental).

ter (RF-530®, Shimadzu, Kyoto, Japan). Measurements were made at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. Recording and evaluation of data were performed using computer integration software (EuroChrom 2000® Knauer).

2.6. Gradient

The program and solvents (automatically degassed using a three-channel degasser, Knauer) used are given in Table 1. The flow-rate was maintained at 1.0 ml/min throughout the experiment using a hydrostatic gradient pump.

Table 1
Chromatographic gradient conditions for the fluorescence HPLC analysis of free intracellular amino acids in PMLs

Duration (min)	Buffers	
	From (% buffer A/B)	To (% buffer A/B)
0–3	97/3	100/0
3–24	100/0	100/0
24–37	100/0	70/30
37–63	70/30	0/100
63–70	0/100	97/3

Buffer A: 19% methanol/81% 0.05 *M* acetate, pH 7.2
Buffer B: 75% methanol/25%, 0.05 *M* acetate, pH 7.2
Flow-rate: 1 ml/min
Pressure: 2900±150 p.s.i.[a] (A: 97%/B: 3%)

[a] 1 p.s.i.=6894.76 Pa.

3. Results

Sampling of the sober volunteers ($n=85$) was performed, on average, at 9.36 a.m. (± 12 min). The average number of PML cells that could be separated from each whole blood sample was $3.49\pm0.82\times10^6$. The detection limit of the described procedure was of the order of $>10^5$ PMLs/sample ($\approx$0.2 ml heparinized whole blood) and, in this respect, was far lower than the average yield of separated PML cells that was achieved. Cell fractionation took 34 ± 4 min, on average. The average purity of the separated cells was $98.8\pm0.8\%$, while their average vitality was $99.1\pm0.6\%$. Cell yields were determined at the same time that vitality was measured. Percentage deviations for duplicate determinations of cell number were $<2\%$. The coefficients of variation (both within- and between-day variations of areas) for 12 (within-day) and ten (between-day) runs of an OPA-derivatized PML sample were between 0.84% (for methionine) and 2.27% (for ornithine; Table 2), and between 1.82% (glutamate) and 4.44% (lysine; Table 3), respectively. Reproducibilities of the retention times (within-day and between-day C.V.) were between 0.02% (lysine) and 0.22% (citrulline; Table 2), and between 0.66% (threonine) and 0.04% (lysine; Table 3), respectively. The average free intracellular amino acid concentrations, normalized with respect to single PML cells, are described in both Table 4 and Fig. 2. A typical elution profile of an OPA-derivatized PML probe is given in Fig. 3.

Table 2
Coefficients of 'within-day' variations (C.V.) of areas respectively mean values and coefficients of 'within-day' variations of retention times [min] given from 12 runs of an OPA-derivatized PML probe

PML probes Amino acid	Abbreviation	Area C.V. (%)	Retention time	
			t (min)	C.V. (%)
Aspartate	(Asp)	1.13	3.29	0.11
Glutamate	(Glu)	1.23	4.48	0.09
Asparagine	(Asn)	1.01	8.28	0.06
Serine	(Ser)	1.24	10.88	0.11
Glutamine	(Gln)	1.19	13.81	0.14
Histidine	(His)	1.35	15.56	0.11
Homoserine	(I.S.)	2.74	19.21	0.17
Glycine	(Gly)	1.82	23.26	0.19
Threonine	(Thr)	1.13	25.87	0.20
Citrulline	(Cit)	1.64	27.13	0.22
Arginine	(Arg)	1.25	32.44	0.13
Taurine	(Tau)	1.17	38.48	0.07
Alanine	(Ala)	1.41	41.31	0.06
Tyrosine	(Tyr)	1.36	44.26	0.05
α-Aminobutyric acid	(Aba)	0.88	49.14	0.04
Tryptophan	(Trp)	1.04	52.79	0.03
Methionine	(Met)	0.84	53.07	0.03
Valine	(Val)	1.30	53.86	0.03
Phenylalanine	(Phe)	0.97	55.42	0.02
Isoleucine	(Ile)	1.30	57.87	0.02
Leucine	(Leu)	2.23	58.86	0.02
Ornithine	(Orn)	2.27	62.33	0.02
Lysine	(Lys)	1.78	64.00	0.02

Table 3
Between-day C.V. of areas and of retention times from ten runs of an OPA-derivatized PML probe

PML probes Amino acid	Abbreviation	Area C.V. (%)	Retention time C.V. (%)
Aspartate	(Asp)	2.76	0.27
Glutamate	(Glu)	1.82	0.41
Asparagine	(Asn)	2.04	0.51
Serine	(Ser)	1.90	0.51
Glutamine	(Gln)	2.72	0.61
Histidine	(His)	2.93	0.59
Homoserine	(I.S.)	2.97	0.63
Glycine	(Gly)	1.98	0.61
Threonine	(Thr)	2.10	0.66
Citrulline	(Cit)	1.93	0.48
Arginine	(Arg)	3.92	0.22
Taurine	(Tau)	4.40	0.17
Alanine	(Ala)	3.95	0.13
Tyrosine	(Tyr)	3.91	0.11
α-Aminobutyric acid	(Aba)	2.64	0.09
Tryptophan	(Trp)	2.27	0.08
Methionine	(Met)	3.79	0.07
Valine	(Val)	3.00	0.07
Phenylalanine	(Phe)	3.46	0.06
Isoleucine	(Ile)	3.73	0.05
Leucine	(Leu)	3.46	0.05
Ornithine	(Orn)	2.91	0.04
Lysine	(Lys)	4.44	0.04

4. Discussion

PML cell fractionation and PML cytolysis were particularly interesting aspects of this study concerning the fluorescence amino acid analysis which followed. Only preparation procedures that prevent

Table 4
Free amino acid concentrations in PML cells from 85 healthy subjects[a]

Amino acid	Abbreviation	Mean (%)	SD (min)	C.V. (%)
Aspartate	(Asp)	2.75	0.49	17.8
Glutamate	(Glu)	5.86	0.73	12.5
Asparagine	(Asn)	0.38	0.05	13.1
Serine	(Ser)	1.51	0.26	17.2
Glutamine	(Gln)	2.47	0.61	24.7
Histidine	(His)	0.69	0.17	24.6
Glycine	(Gly)	2.39	0.37	15.5
Threonine	(Thr)	0.69	0.08	11.6
Citrulline	(Cit)	0.09	0.02	22.2
Arginine	(Arg)	0.27	0.05	18.5
Taurine	(Tau)	35.9	6.85	19.1
Alanine	(Ala)	1.78	0.32	18.0
Tyrosine	(Tyr)	0.29	0.06	20.7
α-Aminobutyric acid	(Aba)	0.21	0.04	19.0
Tryptophan	(Trp)	0.13	0.02	15.4
Methionine	(Met)	0.16	0.03	18.8
Valine	(Val)	0.40	0.05	12.5
Phenylalanine	(Phe)	0.62	0.13	21.0
Isoleucine	(Ile)	0.28	0.04	14.3
Leucine	(Leu)	0.61	0.13	21.3
Ornithine	(Orn)	0.39	0.13	33.3
Lysine	(Lys)	0.58	0.12	20.7

[a] Concentrations are given in pmol/PML cell (mean±SD).

further metabolic activity and thereby reflect the metabolic state at the time of sampling are appropriate for (1) allowing intragranulocyte free amino acid metabolism to be analyzed with a high degree of accuracy and certainty, and (2) detecting small pathophysiological alterations in metabolic processes. Thus, in the preparation of PMLs from whole blood, the goal must be to accomplish the fastest possible separation that retains complete activity, preserves cellular viability (without premature destruction of cellular integrity) and achieves a high degree of cell purity. For this reason, a different procedure to the commonly used Ficoll gradient method was employed for granulocyte separation; this method was a further development of the methods described by Eggelton et al. [20] and Krumholz et al. [3], which allowed very rapid and highly selective enrichment of PMLs from very small quantities of blood. Although we used 4 ml of whole blood, 0.2 ml was sufficient for the isolation of enough granulocytes for HPLC analysis. Cooling of the whole blood samples and of the gradients to 4°C did not have an effect on granulocyte quality, as was confirmed by light microscopy; large numbers of cells could be separated with a high level of, even from small quantities of whole blood, and an average of >99% of the PMLs remained viable, with their cell membranes being mostly intact. Cell yields were determined at the same time that vitality was measured. For duplicate determination, the percentage deviation in the numbers of cells amounted to less than 2%. Our results indicated that it was always necessary to check cell purity and viability to ensure precise and valid PML analysis.

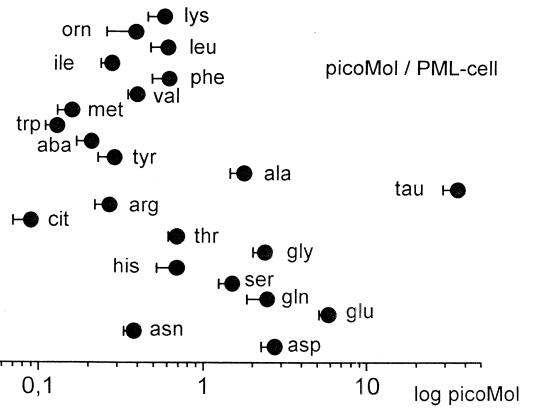

Fig. 2. Free amino acid concentrations (pmol per PML) in PMLs from 85 healthy volunteers. Results are given as the mean±standard deviation. (For abbreviations and intracellular concentrations, see Table 4).

Another objective of the study was to reduced the sample preparation and separation times. This was achieved (compared to other procedures), with an average preparation/separation time of 34 min per sample and, as stated above, all steps of the procedure were done at 4°C. In former studies, separation of PMLs often required more than 1 h (e.g., the procedure of Böyum [21]) and preparation was performed at room temperature or even at 37°C (e.g., Al-Sawaf et al. [8] left the heparinized blood in a water bath at 37°C for 40 min) [9,10,14]. Another problem associated with PML separation arises from the lysis techniques employed in earlier studies, which were sometimes extremely complicated and time-consuming, and did not guarantee any degree of metabolism neutrality. Some investigators employed complicated and very long freezing, thawing or sonication procedures [e.g., Carrea et al. [11] lysed

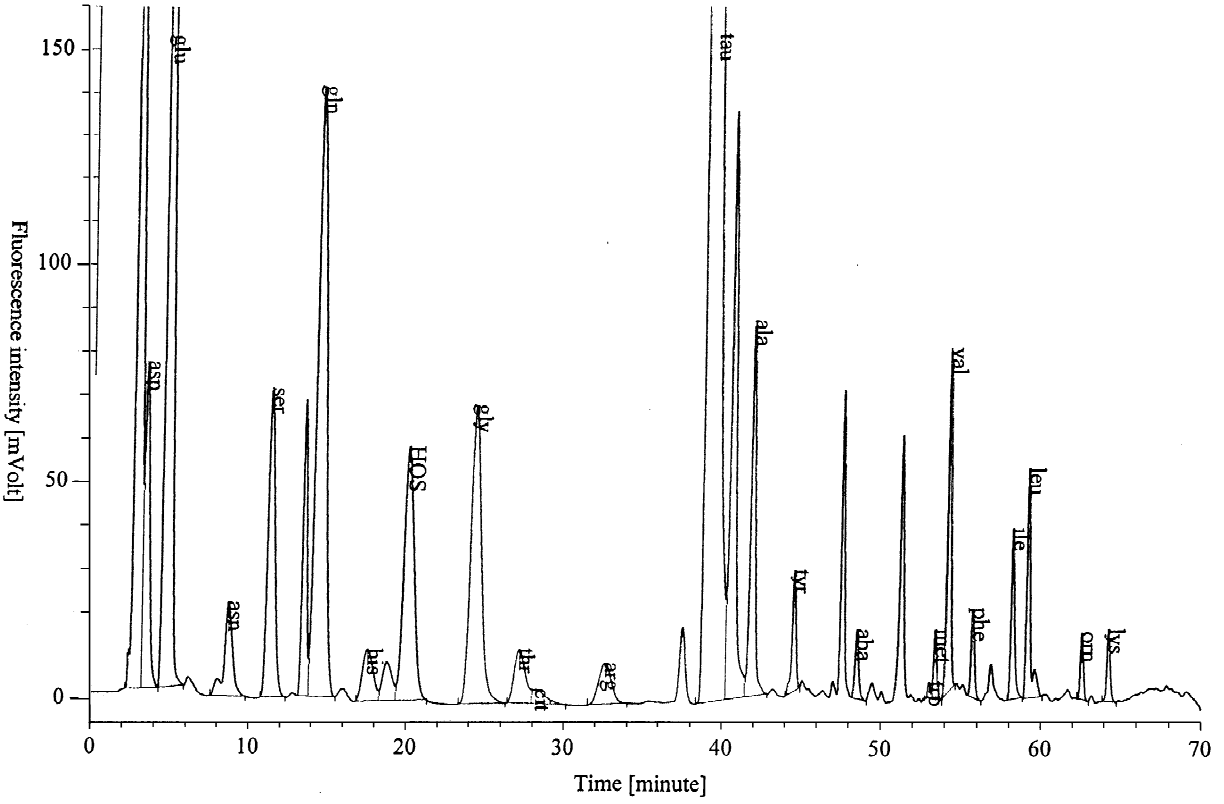

Fig. 3. Typical elution profile of an OPA-derivatized PML probe. PML preparations and chromatographic conditions are described in Section 2. Data recording, evaluation and printing were performed using computer integration software (EuroChrom 2000®).

PMLs using three cycles of freezing (−80°C for 15 min) and thawing (4°C at 60 min)], and some investigators even rewarmed the samples to 37°C or more (e.g., Learn et al. [12] rewarmed the cells to 100°C in a water bath for 1 min to ensure PML cell lysis) and others employed chemical lysis procedures using HCl or KOH solutions [8–11,13–15,22,23]. However, the extent to which cellular metabolism is subject to further alteration after blood sampling with these procedures remains to be studied. In contrast to the studies involving arduous, time-consuming and even chemolytic procedures, our aim was to lyse frozen PML samples in order to prevent further potential amino acid changes during preparation and lysis. Subsequently to PML separation lysis of the frozen (−80°C) PML was performed by freeze drying, a process which in any case was absolutely necessary for allowing long term sample storage (according to the results of Fuchs et al. by human plasma [18]). This allowed for the rapid and complete lysis of the PMLs without the need for additional reagents, which may have had a proteolytic effect, and without the need for rewarming, which could otherwise reactivate cellular activity during the analytical stages.

A further important study goal was to determine amino acid concentrations at the single-cell level. Elsewhere, amino acid concentrations have usually been expressed as μmol/ml or μmol/kg intracellular water (ICW). Cell water is often determined by wet weight−dry weight, and is sometimes corrected for trapped water by determining residual ^{14}C-inulin retention [9,10,14,24] (procedure of Baron and Ahmed [25]). Other normalization parameters include the mass of intracellular soluble proteins [15] and DNA content [26]. Only in a few studies have amino acid concentrations been related to cell number [12,27]. The main problem with the variety of

parameters chosen for normalization is the lack of data comparability; even when different research groups chose the same normalization parameters (e.g. intracellular water), there was no consistency regarding sample preparation, cell fractionation, lysis or the HPLC procedure used. Furthermore, the nature of the pathophysiological mechanisms involved often makes it senseless to compare intracellular amino acid concentrations using biological cellular parameters, since the latter can also become altered during severe disease processes. This applies particularly for the intracellular water parameter. Water can permeate freely through cell membranes (some cells, such as kidney cells, also possess special aquapores for controlling the water balance) so that the physiological intra- and extracellular osmolality, despite differences in electrolyte composition, is virtually the same. However, in severe diseases, there are often pathological changes in water homeostasis and osmolality. In addition, there has been evidence that a lot of drugs used in intensive care units are able to alter cellular ionic channel currents and may lead to changes in intracellular volume regulation [28–33]. Dysregulation of cellular water homeostasis as a result of both balance and distribution disorders can occur, which can affect both the extracellular and intracellular spaces. Apart from separate changes in water homeostasis (hypo- or hypervolemia), osmotic pressure and osmolality (especially changes in sodium concentration) alterations can often cause marked, complex pathophysiological changes (hypotonic/hypertonic dehydration/hyperhydration) associated with fulminant alterations in the intracellular volume of many different cell types (e.g. edema, exsiccosis). Other methodical limitations and non-estimable processes occurring during cell separation (absolute intracellular volume changes during PML separation cannot be observed or measured) that alter intracellular PML water content can also alter methodical accuracy when using the ICW normalization parameter. In addition, there has been evidence that intracellular amino acids have important osmoregulatory functions in regulation of the volume of cells. Taurine in particular, but also glutamate, aspartate and glycine are transported out of (into) cells to achieve a regulatory volume decrease (increase) under anisotonic conditions [34–36].

Intracellular protein mass as a normalization parameter is also associated with a number of problems. Many chronic diseases, and diseases requiring intensive care in particular (post-invasive metabolic alterations after complicated operations, burns, sepsis, liver cirrhosis, nephrotic syndrome, etc.) and drug therapies (i.e. glucocorticoids, β-adrenergic agonists etc.), are associated with severe changes in protein metabolism, such as substantially raised protein catabolism and a significant reduction in intracellular protein mass [37–43]. Pathophysiological changes in protein metabolism, and methodological errors in particular, can lead to dangerous misinterpretations of intracellular amino acid concentrations, especially during continuous surveillance of severe diseases where protein changes are likely to occur. Only with regard to the parameter 'DNA content' can a certain degree of concentration stability be relied upon during the course of a severe disease. As the results of this study show, with careful application of the procedure described here, the intracellular amino acid content can be described accurately (with very low standard deviations) at the single-cell level over a wide range of cell counts per sample. It allows for the accurate, continuous surveillance of severe disease states, especially those that require intensive care. Furthermore, it is not necessary to control for any disease-induced changes in normalization parameters that might alter interpretation of the results.

A major problem with many of the procedures published elsewhere is that cellular stability is hard to guarantee. According to Algermissen et al. [44], both prompt processing of the PML sample, and reliable, safe storage of samples/standards are imperative. Amino acid levels can fall rapidly, with the amides glutamine and asparagine being particularly susceptible to hydrolysis, and samples can also be subject to bacterial degradation. Freezing at −80°C is not sufficient on its own to guarantee long-term stability (>three weeks). Fuchs et al. [18,19,45,46] found that lyophilization (as performed in our study) enables samples to be stored for several months (at −80°C for up to six months) so that concentrations measured are comparable to those made in fresh biological samples. In addition, lyophilization allows for the effective extraction of amino acids from the sample matrix, since the methanolic extraction medium (methanol–water, 80:20, v/v) can dissolve

the protein ligand binding more effectively than both liquid–liquid extraction and protein precipitation involving sulfo-5-salicylic acid (SSA); it is also an ideal solubilization medium for most of the relevant ligands. In addition, the methanol–water mixture (80:20, v/v) can guarantee short-term storage stability (up to 80 h), can act as a vehicle for the internal standards (we used homoserine), and does not extract any protein from the biological samples. For these reasons, the often-recommended deproteinization stage using SSA is not necessary when using methanol. Fuchs et al. [18,19,45,46] showed that chemical preservation using methanol is more effective than physical preservation by cooling to 4°C. After 72 h, all amino acids remained at the 100% level. Matrix-controlled standard calibration is just as important, since amino acid standards prepared in aqueous solutions alone do not behave in the same way as amino acids in a biological sample; hence, similar treatment of standards can only be accomplished using standards prepared in a protein matrix containing isotonic saline. Fuchs et al. [18] showed that the absence of isotonic saline alone reduced the quantity of extraction by up to 70–80%. For reversed-phase HPLC, OPA was chosen as the fluorophor and derivatizing agent. Within a very short period of time, the formation of the isonidol derivative (pH 9.5–10 in 0.5 *M* borate) at room temperature was complete and reproducible. The main advantage of the OPA precolumn derivatization, apart from the low cost, was that the proportion of fluorophor in relation to the amino acid guaranteed a high degree of selectivity and sensitivity. The resulting derivatives were separated on reversed-phases and, because of their small molecular masses, they showed the best separation properties even when (potentially problematic) biological matrices were used. Compared with nitrobenzooxadiazole (NBD), OPA gave better separation properties, but compared to the often-recommended 9-fluorenylmethylchloroformate (FMOC), OPA's advantages included its substantially lower price and the fact that it was not necessary to add pentane to the samples to remove any masking side-products [47,48]. The disadvantage presented by the lower stability of the OPA derivatives (compared to other derivitization procedures) was overcome by exact standardization of the reaction times through utilization of the type of automated, reproducible analytical system described here. Further disadvantages of using OPA as the fluorophor are the low fluorescence intensity in cysteine and cystine derivatives, as well as the fact that secondary amino acids (e.g. proline and hydroxyproline) cannot be determined because of distinct analytical disturbances. In addition, regarding our elution profiles, we found a low resolution of aspartate to corresponding peaks. For this reason, concentrations of aspartate are probably overestimated. A running buffer gradient is also required for optimum separation of the complex amino acid derivative mixture. According to previous results [18], optimum gradient elution of intracellular amino acid mixtures is possible using methanol instead of acetonitrile as the organic phase. Using the column that was applied in this study (other columns produce poorer separations), the selectivity can be further increased.

In summary, the following can be stated: the procedure described here allows for the quantitative, accurate and reproducible analysis of free amino acid concentrations in single PMLs. With regard to the amount of sample required, quantities, the conserving PML separation, sample preparation and stability, as well as the chosen fluorescence HPLC procedure, the described method is superior to methods published elsewhere. Furthermore, it fulfills the strict criteria required for ultrasensitive and comprehensive intracellular amino acid analysis during continuous surveillance in cases of severe disease, and organ dysfunction in particular.

References

[1] U. Staedt, E. Holm, H. Leweling, S. Jacob, J. Striebel, Infusionstherapie 14 (1987) 151.
[2] E. Roth, J. Karner, Infusionstherapie 14 (1987) 147.
[3] W. Krumholz, J. Endrass, G. Hempelmann, Br. J. Anaesth. 75 (1995) 66.
[4] T. Graser, J. Mangold, G. Distler, P. Stehle, P. Fürst, J. Metcoff, Clin. Nutr 5 (Suppl. 72) (1986) 133.
[5] T. Graser, J. Mangold, G. Distler, R. Weber, P. Stehle, J. Metcoff, P. Fürst, ErnährungsUmschau 34 (1987) 88.
[6] M. Cline, Physiol. Rev. 45 (1965) 674.
[7] G. Distler, J. Mangold, R. Weber, T. Graser, P. Fürst, J. Metcoff, Clin. Nutr. 5 (Suppl. 16) (1986) 128.
[8] H. Al-Sawaf, M. Al-Ghamdi, A. AlBekairi, A. Tawfik, Int. J. Immunopharmacol. 15 (1993) 455.

[9] J. Metcoff, J. Am. Coll. Nutr. 5 (1986) 107.
[10] J. Metcoff, R. Lindemann, D. Baxter, J. Pederson, Am. J. Clin. Nutr. 31 (1978) 1627.
[11] A. Carrea, A. Canepa, F. Perfumo, P. Ancarani, E. Verrina, R. Gusmano, Ann. Clin. Biochem. 30 (1993) 559.
[12] D.B. Learn, V.A. Fried, E.L. Thomas, J. Leuk. Biol. 48 (1990) 174.
[13] J. Mårtensson, Metabolism 35 (1986) 118.
[14] P. Fürst, P. Stehle, T. Graser, Infusionstherapie 14 (1987) 137.
[15] A. Canepa, F. Perfumo, A. Carrera, A. Sanguineti, M. Piccardo, J. Chromatogr. 491 (1989) 200.
[16] A. Gitter, L. Heilmeyer, G. Brüschke, in: Taschenbuch klinischer Funktionsprüfungen, 10th ed, Gustav Fischer, Stuttgart, 1978, p. 285.
[17] C.A. Palmerini, C. Fini, A. Floridi, J. Chromatogr. 339 (1985) 285.
[18] M. Fuchs, Diplomarbeit im Fach Biochemie an der Freien Universität Berlin, 1990.
[19] M. Fuchs, Inaugural Dissertation im Fach Biochemie an der Freien Universität Berlin, 1998.
[20] P. Eggleton, R. Gargan, D. Fischer, J. Immunol. Methods 121 (1989) 105.
[21] A. Böyum, Scand. J. Clin. Lab. Invest. 21 (1986) 97.
[22] J. Mårtensson, J. Larsson, G. Nordström, Eur. J. Clin. Invest. 17 (1987) 130.
[23] Y.L. Dong, T. Yan, D.N. Herndon, J.D. Waymack, Surg. Res. 53 (1992) 293.
[24] J. Metcoff, S. Dutta, G. Burns, J. Pederson, B. Matter, O. Rennert, Kidney Int. 24 (Suppl. 16) (1983) 87.
[25] D.N. Baron, S.A. Ahmed, Clin. Sci. 37 (1969) 207.
[26] J. Metcoff, J. Pederson, J. Gable, F. Llach, Kidney Int. 32 (Suppl. 27) (1987) 136.
[27] J. Mårtensson, T. Bolin, Am. J. Gastroenterol. 81 (1986) 1179.
[28] R.O. Law, Biochim. Biophys. Acta 1314 (1996) 43.
[29] P. Espie, A. Devin, B. Guerin, M.J. Rigoulet, Bioenerg. Biomembr. 29 (1997) 591.
[30] P. Geck, B. Pfeiffer, Ann. N.Y. Acad. Sci. 456 (1985) 166.
[31] E. Benoit, Eur. J. Anaesthesiol. 12 (1995) 59.
[32] N. Biljubasic, J. Marijic, V. Berczi, D.F. Supan, J.P. Kampine, Z.J. Bosnak, Anesthesiology 85 (1996) 1092.
[33] P. Charleswoth, G. Pocock, C.D. Richards, J. Physiol. Lond. 481 (1994) 543.
[34] J.E. Olson, R.W. Putnam, J.A. Evers, N. Munoz, Adv. Exp. Med. Biol. 442 (1998) 229.
[35] R.L. Rasmusson, D.G. Davis, M. Liebermann, Am. J. Physiol. 264 (1993) C136.
[36] N. Ballatori, T.W. Simmons, J.L. Boyer, Am. J. Physiol. 267 (1994) G285.
[37] G. Biolo, G. Toigo, B. Ciocchi, R. Situlin, F. Iscra, A. Gullo, G. Guarnieri, Nutrition 13 (1997) 52.
[38] P.O. Hasselgren, J.E. Fischer, Ann. Surg. 225 (1997) 307.
[39] P.Q. Bessey, Z.M. Jiang, D.J. Johnson, R.J. Smith, D.W. Wilmore, World J. Surg. 13 (1989) 465.
[40] M. Rguez-Mariscal, A.S. DelBarrio, J. Larralde, J.A. Martinez, Experientia 49 (1993) 308.
[41] B.K. Tamarappoo, M. Nam, M.S. Kilberg, T.C. Welbourne, Am. J. Physiol. 264 (1993) E526.
[42] S. DelPrato, R.A. DeFronzo, P. Castellino, J. Wahren, A. Alvestrand, Am. J. Physiol. 258 (1990) E 878.
[43] D. Haussinger, E. Roth, F. Lang, W. Gerok, Lancet 341 (1993) 1330.
[44] B. Algermissen, M. Nündel, E. Riedel, GIT Fachzeitschrift für das Laboratorium 33 (1989) 783.
[45] M. Fuchs, E. Riedel, in: GIT, Quantitative Fluoreszenz-HPLC, Analytik von Aminosaüren und Polyaminen, Verlag, Darmstadt, 1995, p. 324.
[46] M. Fuchs, J. Gerlach, J. Encke, J. Unger, M. Smith, P. Neuhaus, E. Riedel, Int. J. Artif. Org. 17 (1994) 663.
[47] P. Fürst, P.L. Pollack, T.A. Graser, H. Godel, P. Stehle, J. Chromatogr. 499 (1990) 557.
[48] H. Engelhardt, Practice of High-Performance Liquid Chromatography, Springer, Heidelberg, 1986, pp. 409.