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Quantitative determination of free intracellular α-keto acids in neutrophils

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Abstract

For the first time, a procedure is described for the quantitative analysis of free α-keto acid content in human neutrophils (PMNs) relative to single cell number by reversed-phase fluorescence high-performance liquid chromatography. The procedure is minimally invasive and is unsurpassed in the quality of PMN separation, ease of sample preparation as well as sample stability. This method can satisfy the rigorous demands for an ultra-sensitive, comprehensive and rapid intracellular α-keto acid analysis in particularly for the surveillance of severe diseases as well as cellular or organ dysfunction.

Keywords: Neutrophils; α-Keto acids

1. Introduction

Procedures for the analysis of free amino acids in human fluids and for studying the intracellular free amino acid pool in differentiated tissues have been the subject of growing clinical interest in the study of metabolic regulatory and pathophysiological phenomena [1–4]. When complex malfunctions of vital cellular and organ systems manifest in severe disease presentation (e.g., sepsis or multiple organ failure), monitoring of extra- and intracellular amino acid metabolism may provide indications for therapeutic interventions [3–7]. Prior studies have established that human polymorphonuclear leukocytes (neutrophils or PMNs) represent a cellular model for investigating intracellular amino acid metabolism [8–10]. PMNs from peripheral blood can be obtained with minimal invasion and far fewer ethical reservations than with the sampling of tissue biopsies. PMNs are recruited in the early stages of an infectious illness and are essential components of the

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human immunological defense against pathogenic agents and therefore represent an ideal cellular ''early warning system'' [9,11].

Measurement of the conventional intracellular amino acids and changes in their transamination or deamination products, the α-keto acids, provide valuable clinically relevant information. In particular α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV) and α-ketobutyrate (KB), the α-keto acids formed from leucine, isoleucine, threonine and methionine, respectively, are significant regulators of proteolysis and protein synthesis in diverse organ cells [12–14]. However, intracellular modifications of the α-keto acids α-ketoglutarate (KG), pyruvate (PYR) or *p*-hydroxy-phenylpyruvate (PPYR) can also provide important information about the cellular energy supply in metabolically superactive cells such as PMNs, since intracellular amino acid metabolism and the tricarboxylic acid cycle are closely linked together [15]. Unfortunately all the methods which have been thus far developed for measurement of extracellular α-keto acids cannot readily be employed for measuring intracellular α-keto acid concentrations [16–25]. The goal of this study was therefore to develop a practical, precise and standardized procedure for analyzing α-keto acids in PMNs.

2. Experimental

The study was approved by the local ethics committee of the Justus Liebig University in Giessen, Germany. Ninety-eight volunteers (57 male, 41 female), aged 23 to 37 years (mean 29 years) with an average height of 172 cm (range 162–191 cm) and mass of 71.5 kg (range 54–96 kg), were selected. Volunteers with metabolic, cardiopulmonary, neurological or allergic diseases, as well as those taking any drugs, were excluded from the study. To exclude potential circadian variations, whole blood samples were taken from each volunteer (12 h of fasting) between 9 and 10 a.m. The blood was drawn into plastic tubes containing heparin (10 I.E./ml), and immediately cooled in an ice water bath before further processing. Sampling was performed on average at 9.3±0.3 a.m. To inhibit blood protease activity, 100 μg/ml phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml antipain (all acquired from Sigma, Deisenhofen, Germany) were added to each tube.

2.1. Separation of polymorphonuclear leukocytes from whole blood

Separation of PMNs was accomplished using a cooled (4 °C) Percoll gradient (Pharmacia, Uppsala, Sweden). Three 4-ml portions (Σ=12 ml) of cooled whole blood from each volunteer were overlaid onto previously prepared and precooled (4 °C) 70%/55% (in 0.9% NaCl) Percoll gradients before centrifugation at 350 *g* for 15 min at 4 °C (Biofuge, Heraeus, Hanau, Germany). This separates the PMNs as a small layer between the erythrocyte and monocyte layers. The PMNs were carefully removed from the sample and suspended in 10 ml cooled (4 °C) phosphate-buffered saline (PBS) stock buffer (diluted 1:10, v/v; 10×PBS stock buffer, without Ca^{2+}/Mg^{2+}, Gibco, Karlsruhe, Germany). 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, Uppsala, Sweden). After 20 s the PMN fraction was immediately brought back to isotonicity by the addition of 1 ml of 2.7% NaCl (Merck, Darmstadt, Germany) at 4 °C and 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 discarded and the PMN fraction again resuspended (200 μl PBS buffer). Subsequently, all PMN fractions were combined and two aliquots of resuspended sample were removed for microscopy. On average, the cell fractionation procedure lasted 34±4 min. Immediately after preparation, the extracted PMN samples were frozen at −80 °C before lyophilization (freeze–dryer CIT-2, Heraeus). These conditions allowed for a PMN lysis which was not chemically mediated and guaranteed longer analyte stability during extended storage of the sample. Samples prepared in this manner were stored at −80 °C until analyzed within a period not exceeding 4 weeks. The purity, determined in duplicate in the first aliquot by dying with ''Türk's Solution'' (Merck) and viability, determined in the second aliquot by exclusion of ''Trypan Blue''

(Merck) were examined and verified by light microscopy (Zeiss, Oberkochen, Germany) [4,10]. Cell yields were determined at the same time that viability was measured, samples with a PMN purity and viability <96% were discarded. In parallel, plasma samples (100 μl) were separated, lyophilized and stored using known techniques [4].

2.2. Preparation of derivatization reagent

For the fluorescence labeling of the α-keto acids, we used *o*-phenylenediamine (OPD, Sigma, Deisenhofen, Germany). Since oxidation of OPD influences the results in a negative way (the oxidized reagent causes variation in the fluorescence intensity) the brown powder must be re-crystallized prior to use [26]. Although the amount of reactive OPD is less when using the oxidized form of the reagent, this re-crystallization procedure is necessary even when starting with the originally supplied substance. The *o*-phenylenediamine was dissolved in heptane at a temperature of 100–120 °C (oil bath, Merck) and the heptane subsequently evaporated in a rotary evaporator (Merck). This procedure yielded a white powder after drying. With storage under N_2 (Sigma) and at 4 °C in a dark bottle, the dry substance is useable for several months. For each batch of analyses, the OPD reagent must be freshly prepared. For each sample, 5 mg of OPD was dissolved in 5 ml of 3 *M* HCl (Sigma) and 10 μl of 2-mercaptoethanol (Sigma) was added to yield OPD-HCl-ME. This reagent solution was stable for several hours without loss of sensitivity [13,27].

2.3. Standard samples

Analytically pure α-keto acids (Sigma) were dissolved in distilled water (Merck) containing 4% human serum albumin (Merck), immediately lyophilized and stored at −80 °C [13,27]. Only the most clinically relevant α-keto acids were measured in the standard samples: KG, PYR, KB, KIC, KIV, PPYR, and KMV (Table 2, Fig. 1).

2.4. Precolumn derivatization procedure

The lyophilizates (PMN, plasma and standard samples) were solubilized in 250 μl of pure methanol (Mallinckrodt Baker, Deventer, The Netherlands). The methanol also contained the α-keto acid, α-ketovalerate (KV; Sigma) as a high-performance liquid chromatography (HPLC) internal standard. KV is a non-physiological α-keto acid. After a 3-min incubation and a 3-min centrifugation step (3000 *g*, Rotixa/KS, Tuttlingen, Germany), 200 μl of the extracts was dried under N_2 (10 min, 20 °C, Messer, Griesheim, Germany). The OPD-HCl-ME reagent (5 ml) was then added, and the samples were incubated for 60 min at 80 °C. The derivatization was stopped after exactly 60 min by cooling for 15 min in ice water. Ethyl acetate (2 ml, Sigma) was added to the samples and mixed for 7 min in an rotary mixer (Merck) to extract the α-keto acids. After extraction, the top ethyl acetate layer was then transferred to a glass vial (2-CRV, Chromacoll, Trumbull, USA). This procedure was repeated twice for each sample. The combined ethyl acetate portions were dried under N_2 (30 min), re-solubilized in 120 μl of methanol and 50 μl of this mixture was injected onto the HPLC column.

2.5. Fluorescence high-performance liquid chromatography

The fluorescence (F)–HPLC system consisted of a pump with a controller for gradient programming (600 E, Waters, Milford, MA, USA) and a programmable autosampler (Triathlon, Spark, The Netherlands) with a Rheodyne injection valve and a 100 μl sample loop (AS 300, Sunchrom, Friedrichsdorf, Germany). A Nova-Pak, 300×3.9 mm I.D., RP-C-18, 60 Å, 4 μm (Waters) analytical column was used for the separation. Column temperatures were maintained at 35 °C using a column oven (Knauer, Berlin, Germany). The column eluent was monitored using a fluorescence spectrophotometer (RF-530, Shimadzu, Kyoto, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 415 nm. Data recording and evaluation was performed using computer integration software (EuroChrom 2000, Knauer). The linear calibration curves were constructed based on area ratios of the standard (St) to the sample (S) chromatograms ($[\text{area}_{\text{keto acid-St}}/\text{area}_{\text{internal standard-St}}]\times$amount or concentration of keto acids injected=calculation factor

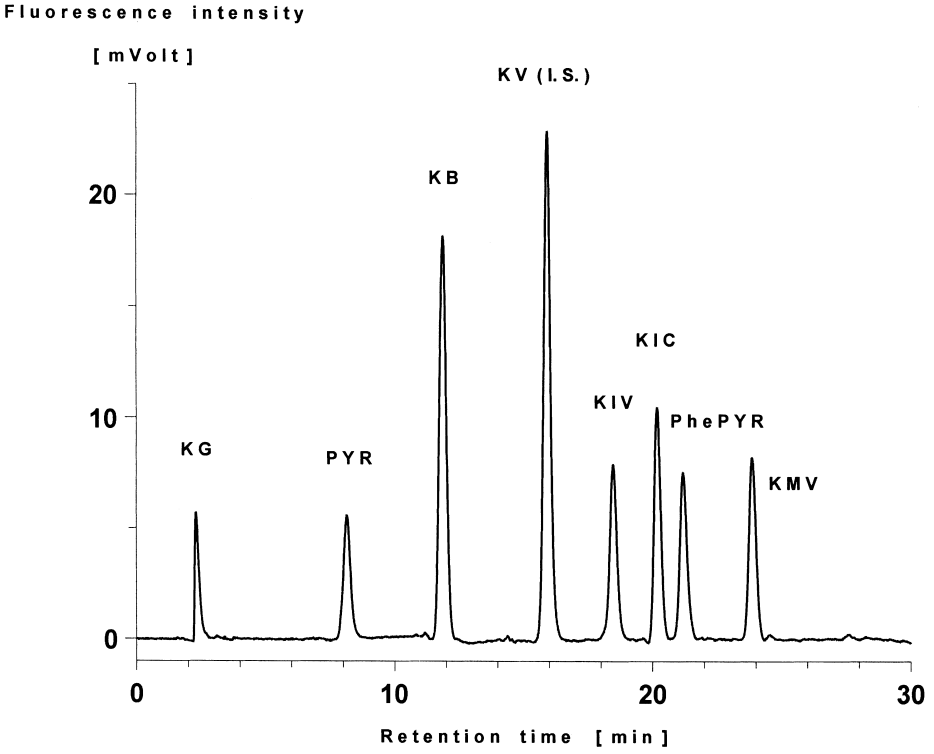

Fig. 1. Typical elution profile of an OPD-HCl-ME-derivatized standard sample.

(CF); [$\text{area}_{\text{keto acid-S}}/\text{area}_{\text{internal standard-S}}$]×CF=final result).

The gradient 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.

Table 1
HPLC gradient conditions

Gradient program					
Step	Time (min)	Interval (min)	Buffer A (%)	Buffer B (%)	Comment
Equilibration		(120 min)	80	20	Pre-run
0	0	0	80	20	Inject
1	0–1	1	53	47	Linear gradient
2	1–16	15	33	67	Linear gradient
3	16–26	10	33	67	Linear gradient
4	26–30	4	80	20	Linear gradient
Buffer A	Methanol–0.05 *M* sodium acetate, pH 7.2 (19:81)				
Buffer B	Methanol–0.05 *M* sodium acetate, pH 7.2 (75:25)				
Flow-rate	1 ml/min				
Pressure	3150±150 p.s.i. (A–B, 80:20)				
Wavelengths	Excitation: 360 nm Emission: 415 nm				

3. Results and discussion

3.1. Precision of the technique, recovery, linearity and detection limit

The average number of PMN cells that could be separated from 12 ml of whole blood was $8.7\pm2.3\times10^6$ cells (mean±SD, $n=98$). The typical measurement range of the described procedure yielding satisfactory sensitivity at approx. 20–100 times of the detection limit (given below) was on the order of 10^6 PMN/sample (≈1.5–2 ml heparinized whole blood) and was lower than the average yield of separated PMN cells. The average purity of the separated cells was 98.6±0.9% and the average viability was 99.0±0.6%. The percent deviation upon duplicate estimations of cell numbers was <5%. Reproducibilities of the retention times were between 0.16 and 0.82%. Moreover, variability of our method, tested with different concentrations of α-keto acid standard samples at the low limit of quantification (LLOQ), 4×LLOQ, at the measurement range and at the upper limit of quantification (ULOQ) also showed satisfactory and reproducible results (Table 2). Our method, therefore, fulfills the current criteria which are demanded in HPLC quality control [28,29].

To estimate the influence of the sample preparation and derivatization process on the measurement of α-keto acids, the recoveries of α-keto acids added to a pooled PMN sample were determined. The recovery values of all α-keto acids examined are shown in Table 3. Every α-keto acid was satisfactorily recovered (79–108%) in comparison with other techniques [16,30–34]. The detector response was linear ($r^2>0.996$) for injected standard samples (50 μl injection volume) in the measurement ranges for all α-keto acid examined. The limits of detection were 0.035 μmol/l (for KIV, KIC, PPYR, KMV; 1.75 pmol/50 μl injection) and 0.125 μmol/l (for KG, PYR and KB; 6.25 pmol/50 μl injection) i.e., well below the physiological range. This sensitivity in detection has not been obtained with other reversed-phase high-performance liquid chromatography techniques using either OPD or another de-

Table 3
Recoveries (%; mean±SD) of α-keto acids added to pooled PMN samples ($n=6$)

	Recovery of α-keto acids added to pooled PMN samples (%)			
	Concentration of keto acids added (μmol/l) ($n=6$)			
	+5	+10	+20	+40
KG	86.3±1.8	89.6±2.5	94.1±3.4	92.7±2.8
PYR	91.9±3.3	102.5±4.7	108.5±5.3	96.8±3.6
KB	89.7±2.6	84.2±3.9	92.3±4.4	87.6±4.0
KIV	83.4±3.8	80.5±2.3	90.8±4.2	85.1±3.7
KIC	79.5±3.6	84.7±3.4	80.2±2.9	87.5±4.4
PPY	92.6±4.1	90.3±4.5	96.8±3.7	90.4±3.9
KMV	88.2±2.7	101.8±4.6	94.5±4.6	104.3±5.1

Table 2
Relative standard deviations (RSDs) of areas at the low limit of quantification (LLOQ), 4×LLOQ, at the measurement range and at the upper limit of quantification (ULOQ) given from six ''within-day'' (WD) and ''between-day'' (BD) runs of an OPD-HCl-ME-derivatized standard sample

	Method variability ($n=6$)											
	LLOQ			4×LLOQ			Measurement range			ULOQ		
	Conc.	RSD (%)		Conc.	RSD (%)		Conc.	RSD (%)		Conc.	RSD (%)	
		WD	BD		WD	BD		WD	BD		WD	BD
KG	$56.5\cdot10^{-12}$	7.8	13.6	$22.6\cdot10^{-11}$	4.5	8.7	$11.3\cdot10^{-10}$	2.1	3.9	$33.9\cdot10^{-10}$	7.8	10.3
PYR	$43.2\cdot10^{-12}$	7.3	11.7	$17.3\cdot10^{-11}$	3.9	6.8	$86.4\cdot10^{-11}$	1.7	3.1	$25.9\cdot10^{-10}$	6.9	9.4
KB	$38.3\cdot10^{-12}$	8.9	10.8	$15.3\cdot10^{-11}$	5.1	7.1	$76.6\cdot10^{-11}$	2.3	3.5	$22.9\cdot10^{-10}$	8.8	12.5
KV (IS)	$3.96\cdot10^{-12}$	11.1	15.9	$1.58\cdot10^{-11}$	5.1	8.6	$7.92\cdot10^{-11}$	2.4	4.1	$2.38\cdot10^{-10}$	6.0	8.7
KIV	$14.1\cdot10^{-12}$	10.1	15.2	$5.64\cdot10^{-11}$	5.9	9.4	$28.2\cdot10^{-11}$	3.5	4.4	$8.46\cdot10^{-10}$	9.7	13.2
KIC	$12.8\cdot10^{-12}$	6.4	12.5	$5.12\cdot10^{-11}$	3.7	6.0	$25.6\cdot10^{-11}$	1.8	2.9	$7.68\cdot10^{-10}$	8.1	12.6
PPYR	$10.5\cdot10^{-12}$	8.5	13.1	$4.20\cdot10^{-11}$	5.3	8.2	$21.0\cdot10^{-11}$	2.2	3.7	$6.30\cdot10^{-10}$	7.5	11.9
KMV	$12.8\cdot10^{-12}$	9.7	14.4	$5.12\cdot10^{-11}$	6.2	9.9	$25.6\cdot10^{-11}$	2.9	4.3	$7.68\cdot10^{-10}$	10.2	14.1

Concentrations (Conc.) of standard samples are given in mol/50 μl injection volume. I.S. = Internal standard

rivatizing reagent as the fluorophor (e.g., 2,3-diaminonaphthalene, 1,2-diamino-4,5-methylenedioxybenzene, etc.) [13,14,16,23,31,34–36].

3.2. Measurement of α-keto acids in human plasma and PMNs

Fig. 2 shows a typical elution profile of OPD-derivatized α-keto acids in a single plasma sample, containing 7.3 μmol/l KG, 77.5 μmol/l PYR, 8.3 μmol/l KB, 13.6 μmol/l KIV, 39.2 μmol/l KIC, 6.9 μmol/l PPYR and 24.8 μmol/l KMV (Table 4). Earlier examinations had found similar values: a mean content of 5–10 μmol/l for KG, 62–82 μmol/l for PYR, 5–12 μmol/l for KB, 12–23 μmol/l for KIV, 33–41 μmol/l for KIC, 4–9 μmol/l for PPYR and 19–27 μmol/l for KMV [16,17,23,31,34,35]. We also observed no differences between females and males in a given age group, similar to previous findings [16,35]. Notably, the plasma α-keto acid levels, excepting the PYR concentrations, were significantly below the typical concentrations of their respective precursor free amino acids [4]. However, the adult population studied here was younger than in other studies, which could explain why the mean α-keto acid values were slightly higher. For example, concentrations of KG, PYR, and branched chain α-keto acids (BCKAs) may be related to a decrease in lean muscular and body mass observed during aging, because most circulating α-keto acids are released through muscle catabolism [37,38]. Further-

Table 4
Free α-keto acid content in PMN cells (mean±SD) and plasma α-keto acid concentrations (mean±SD) from 98 healthy subjects

α-Keto acid ($n=98$)	PMN content (10^{-2} fmol/cell)	Plasma concentration (μmol/l)
KG	1.31±0.70	7.34±2.05
PYR	6.41±3.95	77.5±28.9
KB	4.05±2.79	8.36±1.81
KIV	2.37±1.24	13.6±3.43
KIC	0.37±0.23	29.2±8.24
PPYR	0.34±0.26	6.97±2.74
KMV	0.89±0.67	24.8±7.31

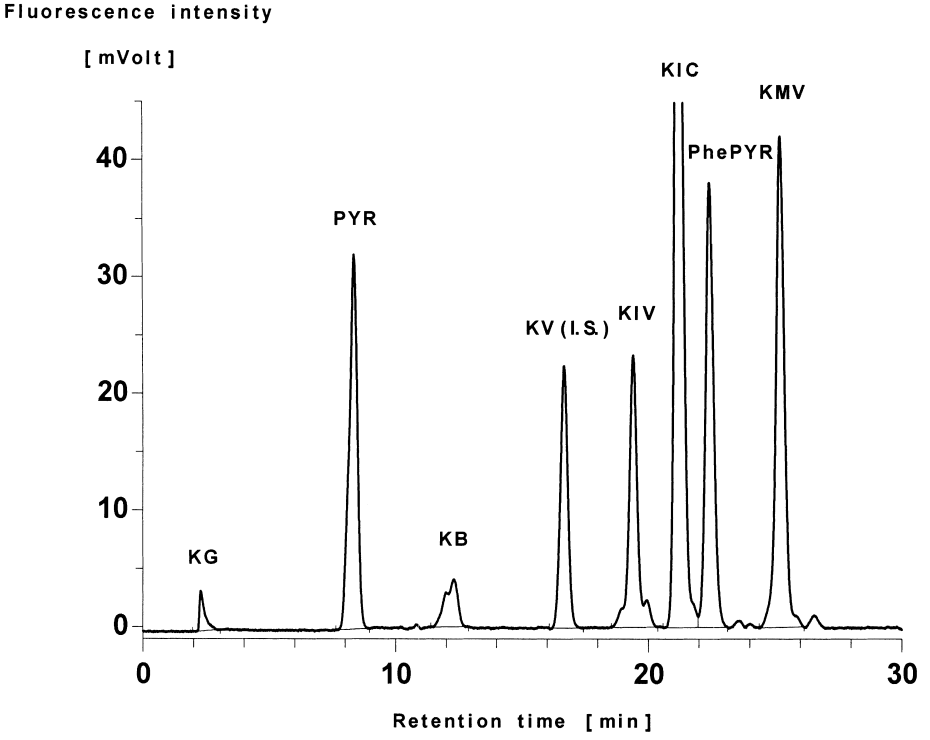

Fig. 2. Typical elution profile of an OPD-HCl-ME-derivatized plasma sample.

more, some disease states cause dramatic changes in albumin levels that could affect circulating BCKA concentrations [39,40]. This can be explained by the fact that BCKAs are extensively bound to albumin. It is therefore useful to determine the reference values of α-keto acids as a function of albumin concentrations. In our study the albumin concentrations were not beyond physiological ranges (42.2±7.3 g/l; reference value in our laboratory: 43±5 g/l) [41].

With regard to the very small amount of sample required for plasma α-keto acid detection our reversed-phase HPLC procedure is superior to methods described elsewhere [14,16,18,23,31,32,34–36].

Our results in Fig. 3 and Table 4 show that this method enables a sufficiently sensitive and specific analysis of the intracellular α-keto acids to be measured in PMNs. As shown in Table 4, the α-keto acid content per cell measured (between 0.34 and $6.41 \cdot 10^{-2}$ fmol/cell) was approximately 20–100× above the detection limits. As we elucidated in previous studies, α-keto acid content in PMNs from healthy individuals are generally approximately a factor of 10 times lower than that of intracellular free primary amino acids [10]. Moreover, there are distinct differences among the individual intracellular α-keto acids in PMNs. Of note is the concentration of PYR in PMN cells: approximately 11 times greater than that of KG. One must also bear in mind that the relative intensities for each α-keto acid will vary based on the extraction yield of its quinoxalinol derivative. Hydrophobic derivates can be extracted with a ~80% yield while hydrophobic derivates are extracted at a much lower yield, approx. 8%. Interpretation of the results should take these considerations into account [13,18,27].

3.3. Methodological considerations

3.3.1. Selective fractionation of PMNs from whole blood

The former techniques used for PMN cell fractionation and PMN cytolysis required modification in

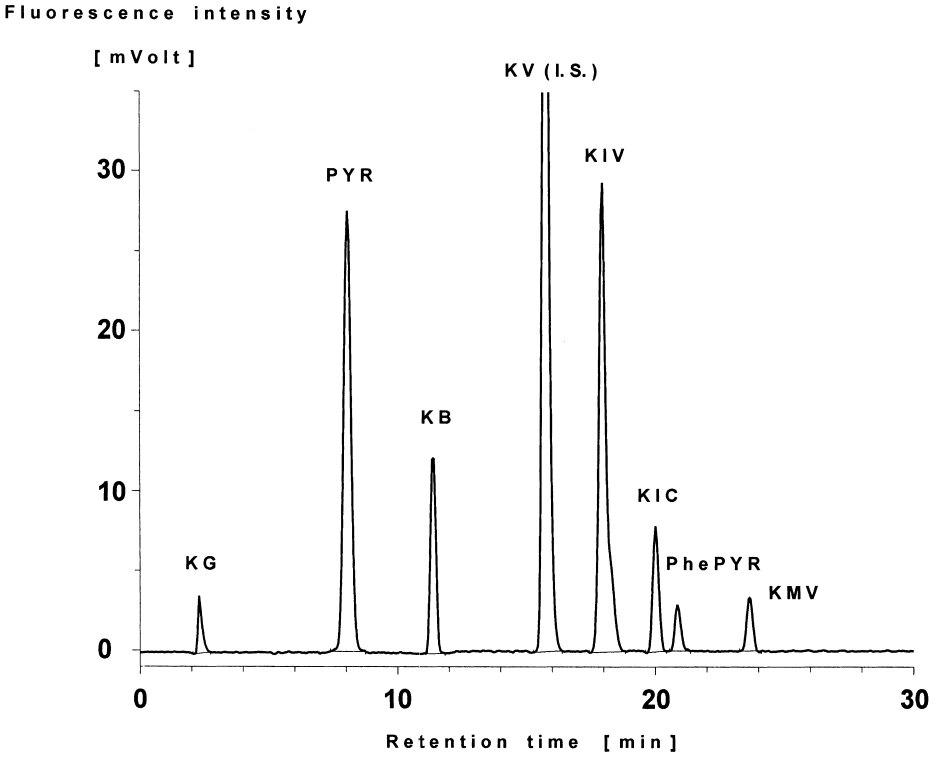

Fig. 3. Typical elution profile of an OPD-HCl-ME-derivatized PMN sample.

terms of speed of cell isolation and yield of viable cells. Only preparation procedures which prevent further metabolic activity and thereby reflect the metabolic state at the time of sampling are appropriate for: (1) allowing intragranulocyte free α-keto acid metabolism to be analyzed with a high degree of accuracy and precision, and (2) detecting small pathophysiological alterations in metabolic processes [6,9]. Thus, the goal of PMN preparation from whole blood must be to accomplish the fastest possible separation that retains complete activity, preserves cellular viability and achieves a high degree of cell purity. For the above reasons, a different procedure was chosen instead of the previously used Ficoll or Dextran gradient methods for granulocyte separation [10]. The Percoll gradient allows a very rapid and highly selective enrichment of PMN from small quantities of blood. Although we used 12 ml of whole blood, 2 ml was sufficient to allow the isolation of enough granulocytes for α-keto acid analysis.

3.3.2. PMN sample preparation technique and lysis procedure

Another important objective was to keep sample preparation times to a minimum. This was achieved with an average preparation time of 34 min per whole blood sample. In former studies, separation of PMN required more than 1 h and preparations were performed under ambient conditions or elevated temperatures [6,9,42,43]. The lysis techniques used in earlier studies were sometimes complicated and time consuming (i.e., very long freezing/thawing cycles, sonication or rewarming steps and chemical lysis procedures) [6,9,42–46]. The rapid and complete lysis of the PMN samples by freeze–drying, without the need for additional reagents, helped prevent sample degradation [10].

3.3.3. Choice of the fluorophor and α-keto acid derivatizing reagent

Keto acids must be derivatized in order to achieve a selective detection. OPD is a specific reagent for α-keto acids to form fluorescence derivatives. At the same time, polar keto acids are transformed to nonpolar compounds that can be separated by reversed-phase HPLC [47,48]. The derivatization reagent was selected so that all keto acids of interest could be separated in one chromatographic run [49].

Earlier investigations on plasma and urine [16,19,23] revealed that very good analytical results could be achieved using OPD derivatization. Fig. 3 and Table 4 show that OPD-based α-keto acids derivatized to highly fluorescent quinoxalinol derivatives enables a very sensitive and specific analysis of the intracellular α-keto acids to be measured in PMN with the analytical column selected. Of particular importance when choosing OPD as the derivatization reagent is the derivatization reagent/sample ratio Samples must be treated with 100–1000-fold excess of OPD in order to achieve rapid and quantitative conversion [20,50]. The choice of derivatization time and temperature must also be controlled as presented herein. An optimum derivatization reaction was produced at a temperature of 80 °C and a reaction time of 60 min. However, the use of OPD as a derivatization reagent still entails methodological problems as presented in the Experimental section.

3.3.4. Stability of α-keto acid derivatives

According to Fuchs et al. [13] and Riedel and co-workers [14,27], both an undelayed processing of the PMN sample and a reliable, safe storage of samples and standards is imperative. For example, α-keto acid levels can fall rapidly due to hydrolysis or oxidation and samples can also be subject to bacterial degradation [13,14,17,51]. Deep freezing alone at −80 °C is not sufficient to guarantee long term stability (>2 weeks). Fuchs et al. [13] and Riedel and co-workers [14,27] found that lyophilization as performed in our study enables subsequent storage of the samples for several months (−80 °C for up to 2 months) so that concentrations measured are comparable to those made in fresh biological samples. In addition, lyophilization allows an extraordinarily effective extraction of the α-keto acids from the sample matrix since the methanolic extraction medium can dissolve the protein ligand binding more effectively than both liquid/liquid extractions and protein precipitations involving 5-sulfo salicylic acid (SSA); it is also an ideal solubilization medium for most of the relevant ligands [13]. In addition, methanol can guarantee short term storage stability (up to 24 h), can act as an vehicle for internal standards and does not extract any

protein from the biological samples [10]. For these reasons, using methanol is an often recommended deproteinization step.

3.3.5. Concentrations of α-keto acids

A further important study goal was to obtain a precise description of α-keto acid concentrations per PMN cell without using normalization parameters (i.e., intracellular water, mass of intracellular soluble proteins, DNA content, etc.) [9,52–54]. The main difficulty with the variety of parameters used for normalization is data comparability, even when different research groups choose the same normalization parameters (i.e., intracellular water). Due to pathophysiological mechanisms observed, most normalization parameters could become altered during severe disease processes or during continuous surveillance of diseases where intracellular water or protein content changes are likely to occur [10]. Moreover, errors caused by variations in sample preparation, cell fractionation, cell lysis or HPLC procedures may lead to incorrect values of intracellular α-keto acid concentrations [13,17]. The data in Tables 3 and 4 show that the intracellular α-keto acid content can be described at the single cell level, i.e., over a wide linearity range. Our method allows for accurate continuous surveillance of severe disease states, particularly those patients requiring intensive care. With careful application of our procedures described here, it is not necessary to control for disease-induced changes in normalization parameters that might alter interpretation of the results.

4. Conclusions

The development of a quantitative and reproducible method for the analysis of important intracellular α-keto acids in PMN presents a substantial methodological challenge due to the high instability and polarity of the α-keto acids being examined. The methods described in this paper have for the first time provided a reproducible and quantitative analysis of metabolically relevant α-keto acids in human neutrophils (PMNs) by reversed-phase F–HPLC. Furthermore, it fulfills the strict criteria required for ultra-sensitive and comprehensive intracellular keto acid analysis in PMNs for the surveillance of severe diseases as well as organ or cellular dysfunction.

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