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REVIEW ARTICLE

Intracellular alpha-keto acid quantification by fluorescence-HPLC

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Abstract Procedures for the analysis of free α-keto acids in human fluids (i.e. plasma, cerebrospinal fluid, urine, etc.) as well as for studying the dynamic free α-keto acid pools in differentiated tissues and organ cells have been the subject of growing clinical interest in the study of metabolic regulatory and pathophysiological phenomena. Due to the high instability and polarity of the α-keto acids being examined, the development of a quantitative and reproducible analysis of metabolically relevant intracellular α-keto acids still presents a substantial methodological challenge. The aim of small sample size, rapid, non-damaging and "metabolism-neutral" cell isolation, careful sample preparation and stability, as well as reproducible analytics technology is not often achieved. Only few of the methods described can satisfy the rigorous demands for an ultra-sensitive, comprehensive and rapid intracellular α-keto acid analysis.

Keywords α-Keto acid analysis · Intracellular · Chromatography · HPLC

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Introduction

Relevance of α-keto acids in the surveillance of complex pathophysiological disorders

The sophisticated quantification of free α-keto 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 or complex pathophysiological functional disorders of vitally important organ systems (Bender 1985; Best 1997; Fuchs et al. 1994; Gerlach et al. 1996; Kielducka et al. 1981; Matsuo et al. 1993; Milley and Sweely 1993; Mühling et al. 2006a, b, c, 2007; Pailla et al. 2000; Roth et al. 1991; Schafer and Schauder 1988; Shestopalov and Kristal 2007; Willems et al. 1978). When complex malfunctions of vital cellular and organ systems manifest in severe disease presentation (e.g. sepsis, multiple organ failure) and a differentiation of the conventional intracellular amino acids and changes in their transamination or deamination products, the α-keto acids may provide additional clinically relevant information to assist in the characterisation of anabolic and catabolic states, as well as for indicating a therapeutic intervention to supplement routine care measures (Aussel et al. 1986; Funchal et al. 2007; Fürst et al. 1992; Grimble 2001; Grimble and Grimble 1998; Hammarqvist et al. 1990, 2001; Mühling et al. 2006a, b, c,

2007; Qureshi 1987; Riedel et al. 1992a, b, 1996, 2000; Walser et al. 1987; Wang et al. 1997; Witko-Sarsat et al. 2000; Young and Bradley 1967) (Fig. 1).

In this respect, the monitoring of intracellular α-keto acids is of particular interest since physiological cell metabolism and basic cell functions rely upon a balanced intracellular α-keto acid content and the cell membrane-mediated separation of cellular α-keto acids from the extracellular plasma α-keto acid pool (Farshidfar 1990; Fuchs 1990, 1998; Fuchs and Riedel 1994; Fuchs et al. 1994; Fürst 1998; Fürst et al. 1992, 1997; Gerlach et al. 1996; Mühling et al. 2006a, b, c, 2007; Riedel et al. 1992a, b, 1996, 2000) (Figs. 2, 3). For example, the considerable α-keto analogues α-ketoglutarate (KG) and pyruvate (PYR) provide important information about the cellular energy supply in metabolically active cells, since intracellular amino acid metabolism and the tricarboxylic acid cycle are closely linked together (Fauth et al. 1990, 1993; Kielducka et al. 1981; Mühling et al. 2006a; Pailla et al. 2000; Willems et al. 1978) (Table 1). Also significant regulators of proteolysis and protein synthesis in diverse organ cells are α-ketobutyrate (KB), α-ketoisovalerianate (KIV), α-ketoisocapronate (KIC), *p*-hydroxy-phenylpyruvate (PPY) and α-keto-β-methylvalerianate (KMV), the α-keto acids formed from alanine, serine, threonine, valine, leucine, phenylalanine, tyrosine or isoleucine (Frexes-Steed et al. 1990, 1992; Fuchs 1990, 1998; Fuchs and Riedel 1994; Fuchs et al. 1994; Fürst et al. 1987, 1992; Gerlach et al. 1996; Mühling et al. 2006b, c; Nissen et al. 1982; Riedel et al. 1992a, b, 1996, 2000; Smeaton et al. 1989; Teigland and Klungsayr 1983a, b) (Table 1).

The long way from amino acid to α-keto acid analysis: a brief historical overview

An important reason underlying the paucity of data for intracellular α-keto acid levels up until now lies within the great methodological problems associated with precise monitoring. This is all the more surprising considering that at the end of the nineteenth century, methods had been developed by Kjeldahl (1883), and a little later also by van Slyke (1911, 1912) which allowed amino acids to be measured non-specifically, both as total nitrogen and as total amino nitrogen. In the search for a more specific detection method, Folin and Denis in 1912 described the calorimetric determination of tyrosine using a phenol reagent. Further calorimetry based analyses followed: arginine was visualized using the Sakaguchi reaction (1925), while histidine was revealed with diazotised sulfanilic acid (Koessler and Hanke 1919; Hanke and Koessler 1920) or by using bromine (Kapeller-Adler 1933). New possibilities opened up at the beginning of the 1940s after the introduction of chromatographic fractionation. Then, it became possible to separate phenylalanine, isoleucine, leucine, proline and valine with the aid of silica gel columns (Martin and Synge 1941). Starch columns even allowed an amino acid separation without the employment

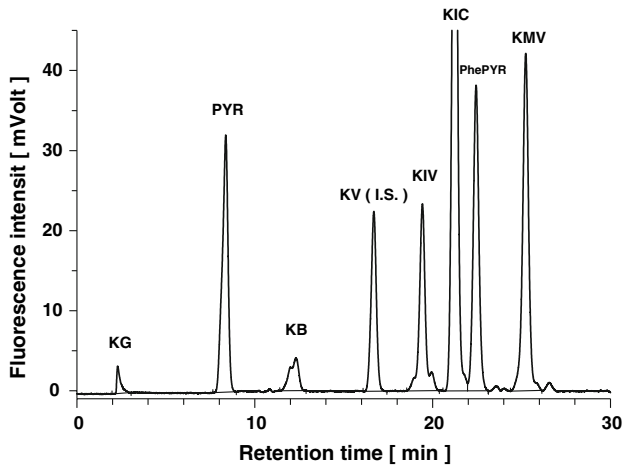

Fig. 1 Typical elution profile of an *o*-phenylenediamine–HCl–ME-derivatized *plasma* sample containing α-ketoglutarate (*KG*), pyruvate (*PYR*), α-ketobutyrate (*KB*), α-ketoisovalerianate (*KIV*), α-ketoisocapronate (*KIC*), *p*-hydroxy-phenylpyruvate (*PhePYR*), α-keto-β-methylvalerianate (*KMV*) and α-ketovalerate (*KV*) as an HPLC internal standard (*I.S.*) (see Farshidfar 1990; Fuchs 1998; Fuchs et al. 1994a; Mühling et al. 2003; Riedel et al. 1992a, b, 2000 for methodological details)

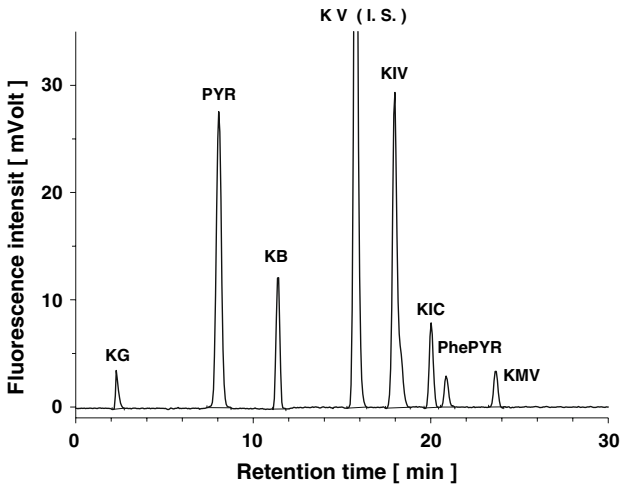

Fig. 2 Example of intracellular α-keto acid monitoring: typical elution profile of an *o*-phenylenediamine–HCl–ME-derivatized *neutrophil* (PMN) sample containing α-ketoglutarate (*KG*), pyruvate (*PYR*), α-ketobutyrate (*KB*), α-ketoisovalerianate (*KIV*), α-ketoisocapronate (*KIC*), *p*-hydroxy-phenylpyruvate (*PhePYR*), α-keto-β-methylvalerianate (*KMV*), and α-ketovalerate (*KV*) as an HPLC internal standard (*I.S.*) (see Fuchs 1998; Fuchs et al. 1994a; Mühling et al. 2003 and Riedel et al. 1992a, b, 2000 for methodological details)

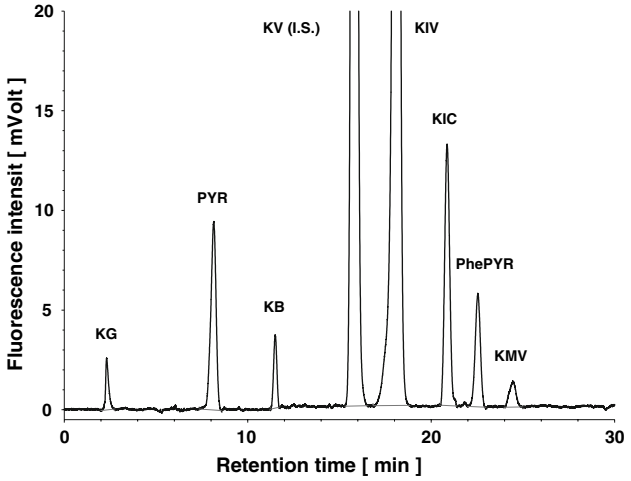

Fig. 3 Example of intracellular α-keto acid monitoring: typical elution profile of an *o*-phenylenediamine–HCl–ME-derivatized *ventricular myocardium mucle cell* sample containing α-ketoglutarate (*KG*), pyruvate (*PYR*), α-ketobutyrate (*KB*), α-ketoisovalerianate (*KIV*), α-ketoisocapronate (*KIC*), *p*-hydroxy-phenylpyruvate (*PhePYR*), α-keto-β-methylvalerianate (*KMV*) and α-ketovalerate (*KV*) as an HPLC internal standard (*I.S.*) (see Mühling et al. 2006c for methodological details)

Table 1 Metabolically relevant amino acids and their transamination or deamination products, the α-keto acids: α-ketoglutarate (KG), pyruvate (PYR), α-ketobutyrate (KB), α-ketoisovalerianate (KIV), α-ketoisocapronate (KIC), *p*-hydroxy-phenylpyruvate (PhePYR) and α-keto-β-methylvalerianate (KMV)

Amino acid	Corresponding α-keto acid
Glutamine, glutamate, alanine, aspartate, histidine, proline	α-Ketoglutarate (KG)
Alanine, serine, threonine	Pyruvate (PYR)
Threonine	α-Ketobutyrate (KB)
Valine	α-Ketoisovalerate (KIV)
Leucine	α-Ketoisocaproate (KIC)
Phenylalanine, tyrosine	*p*-Hydroxy-phenyl-pyruvate (PhePYR)
Isoleucine	α-Keto-β-methylvalerate (KMV)

of an initial derivatization (Synge 1944). Moore and Stein (1948a, b, 1952) continued improving this technique so that they were able to separate the amino acids phenylalanine, leucine, isoleucine, methionine, tyrosine as well as valine in a 4-day run. Finally a refinement of this procedure ultimately allowed the separation of 12 amino acids over 7 days, whereby the subsequent quantification still required a collection and photometric analysis of at least 400 fractions (Stein and Moore 1948).

The key breakthrough in amino acid analysis came at the end of the 1940s with the use of ion-exchange resins and a continuous detection of eluate using ninhydrin for the purposes of "on-line" postcolumn derivatization (Moore and Stein 1948b). Using such an automated system, substantially more free amino acids could be detected even in complex matrices such as plasma, urine or tissue. The analytical times were, however, long (>42 h/sample), and the sample requirements were large (>4 ml protein-free filtrate) (Spackman et al. 1958). A universally applicable analytical instrument was developed in the middle of the sixties in the form of high pressure liquid chromatography (HPLC) which allowed the fractionation of the most varied of substance mixtures with the help of ion exchange, adsorption, partition, affinity or size exclusion chromatography (Horvath et al. 1967; Huber 1969). The development of the so-called reversed-phase-HPLC technique by Kirkland in 1971 represented a substantial step for amino and α-keto acid analysis since this technique allowed a simultaneous separation of polar and homopolar amino and α-keto acids (Kirkland 1971). Fundamentally, the analytical procedure developed by Spackmann, Moore and Stein still represents today the methodological principle for modern α-keto acid analysis, even if current technical (i.e. improved column material, microprocessor-controlled gradient elution and data evaluation, automatic pipetting units as well as highly-sensitive detectors, etc.) and fascinating methodological innovations (reduced sample size, rapid, non-damaging and "metabolism-neutral" cell isolation techniques, careful sample preparation and stability, improved fluorescence-HPLC procedures, etc.) now make it possible to carry out sensitive α-keto acid analyses of the most varied of biological samples in small sample volumes and with shorter analytical times (Algermissen et al. 1989; Early et al. 1984; Engelhardt 1986; Farshidfar 1990; Fuchs 1990, 1998; Fuchs and Riedel 1994; Fuchs et al. 1994; Henschen et al. 1989; Kirkland 2000; Meyer 1986; Mühling et al. 1999, 2003; Riedel et al. 1992a, b).

Methodological requirements for α-keto acid analysis: problem definition

Unfortunately, the methodological requirements for continuous monitoring of free intracellular α-keto acids are very high, especially with regard to the monitoring of diseases and organ dysfunctions requiring intensive care, and only a few procedures can be employed for monitoring. Due to the limited amount of α-keto acids in the cell fraction, the existing sample preparation and derivatization procedures for extracellular fluids require particular adjustment for study of intracellular α-keto acids. The aim of a rapid, non-damaging and "metabolism-neutral" cell or organ tissue isolation is not often achieved with many

procedures (Aussel et al. 1986, 1987; Bender 1985; Best 1997; Brodelius 1984; Early et al. 1984; Farshidfar 1990; Frigerio and Martelli 1973; Hara et al. 1985; Hayashi et al. 1976, 1982, 1983; Holecek 2002; Holecek et al. 1998; Horber et al. 1989; Kallio and Linko 1972 1973; Katrukha and Kukes 1986; Kiba et al. 1989; Kieber and Mopper 1983; Koike and Koike 1984; Livesey and Edwards 1985; Matsukawa et al. 2001; Milley and Sweely 1993; Nakahara et al. 1990; Nissen et al. 1981, 1982; Qureshi 1987; Radeck et al. 1988; Smeaton et al. 1989; Teigland and Klungsayr 1983a, b; Tsuchiya et al. 1983, 1990; Walser et al. 1987, 1989; Wang et al. 1988, 1997; Woolf et al. 1982). Such a procedure must preserve the status quo of the α-keto acid content during the sampling, and must result in the retention of full cellular viability (without prematurely destroying cellular integrity during the purification), as well as a high state of purity.

Another significant problem is the lack of comparability between previously published experimental findings. This is due partly to the variety of techniques employed for cell separation, but also and most significantly, to the variety of ways in which the intracellular α-keto acid concentrations are described (Aussel et al. 1986, 1987; Bender 1985; Bennet et al. 1993; Best 1997; Brodelius 1984; Early et al. 1984; Farshidfar 1990; Fuchs 1998; Fuchs and Riedel 1994; Fuchs et al. 1994; Funchal et al. 2007; Fürst et al. 1987, 1992; Gerlach et al. 1996; Hara et al. 1985; Hayashi et al. 1976, 1982, 1983; Henschen et al. 1989; Horber et al. 1989; Huber 1969; Kiba et al. 1989; Kiedulka et al.1981; Koike and Koike 1984; Meyer 1986; Milley and Sweely 1993; Mühling et al. 1999, 2003; Nissen et al. 1981, 1982; Pailla et al. 2000; Qureshi 1987; Radeck et al. 1988; Riedel et al. 1992a, b, 2000; Schafer and Schauder 1988; Smeaton et al. 1989; Teigland and Klungsayr 1983a, b; Tsuchiya et al. 1983, 1990; Walser et al. 1987, 1989; Wang et al. 1988, 1997; Willems et al. 1978; Woolf et al. 1982). Relating the concentrations to a biological parameter which can itself become changed during severe diseases, or which can be subject to acute changes in other disease courses, is fraught with essential problems. Another problem is that many methods are inadequate regarding sample preparation (e.g. cytolysis, protection from hydrolysis, amino acid extraction, etc.) and maintenance of sample stability (long term storage and stability before HPLC analysis) (Algermissen 1989; Farshidfar 1990; Fuchs 1990, 1998; Mühling et al. 1999, 2003; Riedel et al. 1992a, b, 1996, 2000). The choice of fluorescence-HPLC procedure is also of great importance for an appropriate and exact amino acid analysis (Farshidfar 1990; Fuchs 1990, 1998; Riedel et al. 1992a, b). The goal of this method overview is, therefore, to compare and describe comprehensive and highly precise procedures for analyzing intracellular α-keto acids which fulfil the demands for the continuous surveillance of the most severe forms of disease.

Methodological considerations

Cell fractionation, preparation and cytolysis

As addressed in the problem definition, the cell fractionation and cytolysis should be particularly interesting aspects concerning the fluorescence amino acid analysis which followed. Only preparation procedures, which prevent further metabolic activity and thereby reflect the metabolic state at the time of sampling, are appropriate for (1) allowing intracellular free α-keto acid metabolism to be analysed with a high degree of accuracy and precision, and (2) detecting small pathophysiological alterations in metabolic processes. Another important objective is to keep sample preparation and separation times to a minimum. Unfortunately, many of the scientific results published so far remain contestable because incorrect methodologies were selected or fundamental analytical errors were committed (e.g. cell separation procedures which require more than 1 or 2 h, preparation was performed at room temperatures or even at 37°C or higher, etc.) (Al Sawaf et al. 1993; Böyum 1983, 1984; Canepa et al. 1989, 2002; Carrea et al. 1993; Fürst et al. 1987, 1992; Mårtensson 1986; Mårtensson et al. 1987; Metcoff 1986; Metcoff et al. 1989). Further, special problems may also arise from the lysis techniques employed. In the past these techniques were sometimes extremely complicated and time-consuming, and often did not guarantee any degree of metabolism neutrality (e.g. complicated and very long freezing, re-warming, thawing or sonication procedures, chemical lysis techniques using HCl or KOH solutions, etc.) (Al-Sawaf et al. 1993; Böyum 1983, 1984; Metcoff 1986; Metcoff et al. 1989; Learn et al. 1990; Mårtensson 1986; Mårtensson et al. 1987). For example, Carrea et al. (1993) lysed the cells by three cycles of freezing (−80°C for 15 min) and thawing (4°C at 60 min); Learn et al. (1990) re-warmed the cells to 100°C in a water bath for 1 min for PML cell lysis. However, the extent to which cellular metabolism is subject to further alteration after sampling with these procedures remains to be studied.

In contrast to the studies involving arduous, time consuming and even chemolytic procedures, current investigations showed that the lysis of the frozen samples is beneficial in preventing further potential α-keto acid changes during preparation and lysis (Farshidfar 1990; Fuchs 1990, 1998; Mühling et al. 1999, 2003, 2006c; Riedel et al. 1992a, b, 1996, 2000). Here, lysis of the frozen (−80°C) cells was performed by freeze drying, a process which in any case seems to be absolutely necessary for allowing long-term sample storage up to 4 weeks. For example, Mühling et al. (2003) separated polymorphonuclear leukocytes (PMN) using a cooled (4°C) Percoll®-gradient. Portions of cooled whole blood were

overlaid onto previously prepared and precooled (4°C) 70/55% (in 0.9% NaCl) Percoll®-gradients before centrifugation at 4°C. This separates the PMN as a small layer between the erythrocyte and monocyte layers. The PMN were carefully removed from the sample and suspended in cooled (4°C) phosphate buffered saline (PBS) stock buffer. After a second centrifugation step, PBS buffer was discarded and the erythrocytes remaining in the sample were hypotonically lysed using cooled (4°C) distilled water. After 20 s the PMN fraction was immediately brought back to isotonicity and resuspended by adding diluted stock PBS buffer. After a third centrifugation step the PBS buffer was discarded and the PMN fraction again resuspended. Subsequently, all PMN fractions were combined and aliquots of resuspended sample were removed for microscopy (the cell fractionation procedure lasted $\approx$34 min). Immediately after preparation, the extracted PMN samples were frozen at −80°C before lyophilisation [the last steps were also successfully applied in ventricular myocardium muscle cells (Mühling et al. 2006c)]. These conditions allowed for a cell lysis that 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 analysed within a period not exceeding 4 weeks. In our opinion, techniques which especially ensure a rapid and complete lysis of the frozen cell samples (e.g. by freeze drying), without the need for additional reagents and rewarming which may act proteolytically or could otherwise reactivate cellular activity during the preparation steps may help prevent sample degradation and, therefore, provide crucial advantages. Moreover, cooling of the cell samples and the gradients to 4°C did not impede cellular quality, however, this proceeding demands strict checks of cell purity and viability (i.e. by light microscopic controls of each sample, etc.) for ensuring a precise and valid intracellular free α-keto acid analysis (Algermissen 1989; Farshidfar 1990; Fuchs 1990, 1998; Gerlach et al. 1996; Mühling et al. 1999, 2003, 2006a, b, c, 2007; Riedel et al. 1992a, b, 2000).

Choice of the α-keto acid fluorescence derivatizing reagent

In order to achieve a selective detection, α-keto acids must first be derivatized because at the same time the polar keto acids are transformed to nonpolar compounds that can be separated by reversed-phase HPLC. Former studies described in detail that the choice of derivatizing reagent is critical for optimising reproducibility, accuracy, sensitivity and for efficiency of the analysis (Aussel et al. 1986, 1987; Hayashi et al. 1976, 1982, 1983; Henschen et al. 1989; Horber et al. 1989; Matsukawa et al. 2001; Nissen et al. 1981, 1982; Pailla et al. 2000; Riedel et al. 2000, Smeaton et al. 1989; Walser 1987, 1989; Wang et al. 1988). Therefore, the major aim is to select an optimal derivatization reagent so that all α-keto acids of interest can be separated in one chromatographic run only. As Teigland et al. (1983a, b), Katrukha and Kukes (1986), as well as Farshidfar (1990) have shown, 2,4-dinitrophenylhydrazine (DNP), one of the first reagents employed for precolumn HPLC derivatization, is associated with substantial analytical problems (even gas chromatographic procedures accommodating DNP-derivatization provide no satisfactory analytical results). Kallio and Linko (1972, 1973) as well as Farshidfar (1990) found that a gas chromatographic separation of DNP α-keto acid derivatives requires a second derivatization step, since due to the persistence of OH groups, an ideal volatility of the derivatization products can still not be achieved. Therefore, improved methods developed later stemmed from the fact that the existing methods for analytical RP-HPLC as well as gas chromatography incorporating DNP derivatization were not sufficiently accurate for making quantitative intracellular estimations (Aussel et al. 1987; Fuchs 1998; Fuchs et al. 1994; Gerlach et al. 1996; Mühling et al. 2003, 2006c; Pailla et al. 2000). The detection thresholds of these procedures are in the nanomole range and, therefore, approximately 100-fold higher than those required for a precise analysis of intracellular α-keto acids.

In light of the assumption that intracellular free α-keto acid levels would be present only in very small amounts, as well as the demand for higher detection thresholds, sensitivity, etc., some scientists opted to choose the methods first described by reversed-phase HPLC combined with *o*-phenylenediamine (OPD) derivatization for separating the α-keto acids (of all carbon acids present in the sample, OPD is a selective reagent in that it can only react with α-keto acids form fluorescence derivatives) (Farshidfar 1990; Fernandes et al. 1986; Fuchs 1998; Fuchs et al. 1994; Hayashi et al. 1976, 1982, 1983; Hockenhull and Floodgate 1952; Koike and Koike 1984; Matsukawa et al. 2001; Pailla et al. 2000; Riedel et al. 1992a, b, 1996, 2000; Rocchicciolli et al. 1981; Wolff et al. 1982). These investigations showed that OPD-based α-keto acids derivatized to highly fluorescent quinoxalinol derivatives (a) were qualitatively and quantitatively recorded and showed no perturbing effects and (b) enabled a very sensitive and specific analysis of intracellular α-keto acids with the analytical column selected. Moreover, by using column material with a small bead diameter and a high packing density of the silica gel stationary phase (C18 silanes), the resolution of the system could be improved and a high elution resolution guaranteed, even as separation times were decreased.

Of particular importance when choosing OPD as the derivatization reagent is the derivatization reagent to sample ratio. According to Woolf et al. (1982) as well as Kieber and Mopper (1983), quinoxalinol derivatives must be produced with an excess of OPD (100–1,000-fold) in order to achieve a rapid and quantitative conversion. According to previous results, the choice of derivatization time and temperature must also be adhered. An optimum derivatization reaction [consistent with the findings of Farshidfar (1990) in human plasma] was described at a temperature of 80°C and a reaction time of 60 min (Fuchs 1998; Fuchs and Riedel 1994; Gerlach et al. 1996; Mühling et al. 2003, 2006c; Riedel et al. 1992a, b, 1996, 2000). However, the use of OPD as a derivatization reagent still entails methodological problems. OPD is extremely photo- and oxidation-sensitive, and potential fluorescent by-products of the oxidized OPD considerably disrupt analysis due to their similar retention times. In order to prevent such unwanted side reactions, OPD must be re-crystallised out with *n*-heptane at temperatures of 100–120°C in an oil bath before use and, after the heptane has been subsequently evaporated, stored in the dark under inert (N_2) conditions at 2–8°C for repeated analyses. Therefore, the OPD reagent must be freshly prepared for each batch of analyses (OPD is here mostly dissolved in 3 M HCl and 2-mercaptoethanol) (Fuchs 1998; Fuchs and Riedel 1994; Gerlach et al. 1996; Katrukha et al. 1986; Livesey et al. 1985; Mühling et al. 2003, 2006a, b, c, 2007; Riedel et al. 1992a, b, 1996, 2000). For example, Fuchs (1998), Fuchs and Riedel (1994) and Mühling et al. (Mühling et al. 2003, 2006b) solubilised the lyophilizates in pure methanol which also contained the α-keto acid, α-ketovalerate, as an HPLC internal standard. After incubation followed by a centrifugation step the extracts were dried under N_2. The OPD–HCl–mercaptoethanol reagent 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 was added to the samples to extract the α-keto acids. After extraction, the top ethyl acetate layer was then transferred to a glass vial (this procedure was repeated twice for each sample). The combined etylacetate portions were dried under N_2, re-solubilized in methanol and was injected onto the HPLC column.

Detection of α-keto acids with OPD: are there any analytical advantages by using gas chromatography?

With respect to the studies of Early et al. (1984), Fernandes et al. (1986), Frigerio and Martelli (1973), Rocchiccioli et al. (1981) as well as Wolf et al. (1982), gas chromatographic separation of quinoxalinol derivatives, especially, are often fraught with major analytical problems. Just as with the DNP derivatization of α-keto acids, a second derivatization step was required which further complicated the analysis. Although the groups mentioned above showed that a gas chromatographic separation could be successfully carried out by additionally derivatizing the OH groups (silylation of 3-alkyl-quinoxalinol to *o*-tri-methyl-silyl-3-alkyl-quinoxalinol), the resulting nonpolar silylation products are not sufficiently stable due to changes in their molecular conformation. According to the results of Frigerio et al. (1973), decomposition (tautomeric transformation) from *o*-trimethylsilyl-*N*-trimethylsilyl-quinoxalinol occurs after approximately 30 min, making a serial investigation of repeated samples impossible. Farshidfar (1990), however, described a gas chromatographic procedure that guarantees a qualitatively satisfactory separation of quinoxalinol derivatives without silylation, although this procedure is also not suitable for intracellular measurements since the required quantitative accuracy and sensitivity cannot be guaranteed at the concentrations of derivatives present.

Stability of α-keto acid fluorescence derivatives

Another important methodological point is to guarantee α-keto acid stability. For example, α-keto acid levels can fall rapidly due to hydrolysis or oxidation. Samples can also be subject to bacterial degradation. According to both Farshidfar (1990) and Riedel et al. (1992a, b, 1996, 2000), an undelayed processing of the PMN sample and a reliable, safe storage of samples and standards is imperative. Deep freezing alone at −80°C is not sufficient to guarantee long-term stability (>2 weeks). Farshidfar (1990) and Riedel et al. (1992a, b) also found that lyophilisation enables subsequent storage of the samples for several months (at −80°C for up to 2 months). Moreover, they also showed that concentrations measured were comparable to those made in fresh biological samples. However, lyophilisation also 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), but it seems an ideal solubilization medium for most of the relevant ligands too (Fuchs 1990, 1998; Fuchs and Riedel 1994; Fuchs et al. 1994; Koike and Koike 1984; Mühling et al. 1999, 2003, 2006a, b, c, 2007). In addition, methanol can guarantee short-term storage stability (≤24 h), can act as a vehicle for internal standards (e.g. α-ketovalerate) and does not extract any protein from the biological samples. Therefore, using methanol is an often-recommended deproteinization step.

Moreover, an optimal running buffer gradient is also required for separating the complex α-keto acid derivative mixture. According to former findings, an optimum

gradient elution of intracellular α-keto acid mixtures is possible through the use of methanol, instead of the often used acetonitrile, as the mobile phase (Fuchs 1998; Fuchs et al. 1994; Koike and Koike 1984; Mühling et al. 2003; Riedel et al. 1992a, b; Teigland and Klungsayr (1983b). Another analytical advantage is a good solubility and stability when using methanol acetate buffers. According to Farshidfar (1990), the optimum acetate ion concentration here is 50 mM. Higher acetate ion concentrations alter the elution time, so that the analysis time is substantially increased.

Description of α-keto acid concentrations

A further scientific goal of special importance is to obtain a precise description of α-keto acid concentrations measured. In principle there are several options, but some of these possess huge analytical risks. One possibility is to express the free α-keto acid quantities measured in µmol/ml or µmol/kg intracellular water. As such, cell water is often determined by wet weight–dry weight, sometimes corrected for trapped water by determining residual ^{14}C-inulin retention (procedure according to Baron and Ahmed; 1969) (Metcoff 1986; Metcoff et al. 1989; Mühling et al. 2006c). Other normalisation parameters include the mass of intracellular soluble proteins, DNA content, or the relation to single cell numbers (Canepa et al. 1989; Fuchs 1990, 1998; Fuchs and Riedel 1994; Fuchs et al. 1994; Fürst et al. 1987; Learn et al. 1990; Mårtensson 1986; Mårtensson et al. 1987; Metcoff 1986; Metcoff et al. 1989; Mühling et al. 1999, 2003, 2006a, b). The main problem with the variety of parameters chosen for normalisation is the lack of data comparability: even when different research groups chose the same normalisation parameters (e.g. wet weight–dry weight, intracellular water, protein content etc.) there was often no methodological consistency (i.e. regarding sample preparation, cell fractionation, lysis, or the HPLC procedure used).

On the other hand, the nature of the pathophysiological mechanisms involved often makes it senseless to compare intracellular α-keto acid concentrations using biological cellular parameters, since the latter can also become altered during severe disease processes. This applies particularly for the protein mass or intracellular water parameters. For example, diseases requiring intensive care or chronic illness in particular as well as many drug therapies frequently are associated with severe changes in protein metabolism which can be manifested by a substantially raised protein catabolism and a significant reduction in intracellular protein mass. During continuous surveillance of severe diseases where protein changes are likely to occur, especially, pathophysiological changes in protein metabolism and/or methodological errors in particular, they may lead to dangerous misinterpretations of intracellular α-keto acid concentrations (Andrews and Griffiths 2002; Biolo et al. 1997; Delano et al. 2006; Engel et al. 2006; Hasselgren 2000; Hasselgren and Fischer 1998; Pereira et al. 2005a, b; Reid 2004). However, intracellular water as a normalisation parameter is also fraught with a number of problems. Dysregulation of cellular water homeostasis as a result of both balance and distribution disorders can affect both the extracellular and intracellular spaces because water can permeate freely or via aquapores through cell membranes so that the physiological intra- and extracellular osmolality, despite different electrolyte composition, is virtually the same (Alexander and Grinstein 2006; Chen and Kempson 1995; Lang et al. 1998; Law 1991; Strange 2004; Trachtman 1991; Verbalis 2006; Yancey 2005). Apart from separate changes in water homeostasis, osmotic pressure, and osmolality, alterations can often cause marked complex pathophysiological changes (hypotonic/hypertonic dehydration/hyperhydration) associated with fulminant alterations in the intracellular volume (e.g. edema, exsiccosis). However, in severe diseases, there are often pathological changes in water homeostasis and there has been evidence that a lot of drugs used in intensive care are able to alter intracellular cellular volume regulation too (Bennet et al. 1993; Bender 1985; Fürst 1998; Fürst et al. 1992; Frexes-Steed et al. 1990, 1992; Henschen et al. 1989; Kielducka et al. 1981; Metcoff et al. 1989; Riedel et al. 1992a, b, 1996, 2000; Roth et al. 1987; Verbalis 2006).

In addition, there has been evidence that intracellular amino and α-keto acids also have important osmoregulatory functions in volume regulation of cells. In particular, taurine and neutral amino acids, but also some α-keto acids (e.g. α-ketoisocaproate) are transported into or out of the cells to achieve a regulatory volume increase/decrease under anisotonic conditions (Alexander and Grinstein 2006; Baron and Ahmed 1969; Baumgartner 1993; Baumgartner et al. 1993; Chen and Kempson 1995; Holecek 2002; Holecek et al. 1998; Lang et al. 1998; Law 1991; Mårtensson 1986; Mårtensson et al. 1987; Metcoff 1986; Roth et al. 1991; Verbalis 2006; Walser et al. 1989; Yancey 2005). And at least, other methodical limitations and non-estimable processes occurring during cell separation that alter intracellular water content may also alter methodical accuracy when using the ICW normalization parameter (Baron and Ahmed 1969; Fürst et al. 1987; Metcoff 1986; Metcoff et al. 1989). Only with regard to the parameter "DNA content" can a certain degree of concentration stability be relied upon during the course of severe diseases (Johnson and Metcoff 1986; Metcoff 1986). Interestingly, current investigations showed that the intracellular α-keto acid content can also be described precisely at the single cell level with high recovery and over a wide linearity range and may, therefore,

allow for accurate continuous surveillance of severe disease states, particularly those patients requiring intensive care (Learn et al. 1990; Mårtensson 1986; Mühling et al. 1999, 2003). Furthermore, with careful application of this technique, it seems to be unnecessary to control for disease-induced changes in normalisation parameters that might alter interpretation of the results.

Conclusions

Methods for a quantitative and reproducible analysis of important α-keto acids in human fluids (i.e. plasma, cerebrospinal fluid, urine, etc.) as well as for studying the dynamic free α-keto acid pools in differentiated tissues or organ cells still presents a substantial methodological challenge due to the high instability and polarity of the α-keto acids being examined. As discussed in this paper, the aim of small sample size, rapid, non-damaging and "metabolism-neutral" cell isolation, careful sample preparation and stability, as well as reproducible analytics technology (i.e. using sophisticated reversed-phase fluorescence high-performance liquid chromatography procedures) is not often achieved. Only few of the methods described can satisfy the rigorous demands for a sensitive, comprehensive and rapid intracellular α-keto acid analysis for the careful surveillance of severe diseases as well as organ or cellular dysfunction.

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