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Note

Quantification of branched-chain α-keto acids as quinoxalinols: importance of excluding oxygen during derivatization

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The quantitative analysis of branched-chain α-keto acids (BCKA), the first intermediates in the oxidation of the essential amino acids L-leucine, L-isoleucine and L-valine in mammals, is of considerable widespread interest [1—17]. Several methods have been developed for this using gas chromatography (GC) [1—8], high-performance liquid chromatography (HPLC) [9—12], or enzymatic determination [13]. The last is rapid and sensitive but does not distinguish between α-keto-β-methylvalerate, α-ketoisocaproate and α-ketoisovalerate. In our experience the HPLC method of Hayashi and co-workers [9, 10] with fluorescence detection is highly sensitive and has a single derivatization procedure yielding stable quinoxalinols. Quinoxalinol formation is also frequently used with GC methods of BCKA analysis prior to a secondary derivatization with bis(trimethylsilyl)acetamide (BSTFA) to more volatile products [1, 2, 4, 6—8].

Over a period of several weeks we obtained standard curves using procedures described by Hayashi et al. [9] but observed variations in the ratio of the peak areas for each BCKA to that for the internal standard, α-ketooctanoic acid. The presence of oxygen during derivatization was found to have a considerable effect and this report demonstrates that exclusion of oxygen improves the reliability of the derivatization procedure and gives a significant increase in the sensitivity of the method for α-ketoisovaleric acid.

EXPERIMENTAL

Preparation of quinoxalinols

Sodium salts of D,L-α-keto-β-methylvaleric (KMV), α-ketoisocaproic (KIC) and α-ketoisovaleric (KIV) acids and the free α-ketooctanoic acid (KOA)

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(Sigma, London, U.K.) were dissolved together in water each at a concentration of 50 nmol ml^{-1} and frozen at −20°C. Solutions of *o*-phenylenediamine were prepared daily by dissolving 40 mg of the dihydrochloride (Sigma) and 100 μl of mercaptoethanol in 20 ml of 2.0 *M* hydrochloric acid. Nitrogen (oxygen-free) and oxygen (containing 5% carbon dioxide) were from BOC. The α-keto-acid solution (0.5 ml) and *o*-phenylenediamine solution (2.0 ml) were mixed in 160 × 16 mm screw-capped Sovirel tubes and heated at 80°C for 2 h. The tubes were cooled with a large volume of water at approx. 15°C. Saturated sodium sulphate solution (4 ml) and ethyl acetate (5 ml) were added and the quinoxalinols extracted into the upper, organic phase by shaking for 5 min. Almost all the upper phase was pipetted onto anhydrous sodium sulphate (approx. 100 mg) for drying at 1—4°C overnight before being evaporated to dryness. For chromatography, the residue was dissolved in dimethylformamide (40 μl) and water (100 μl) and 20—40 μl aliquots were injected onto the column.

The BCKA were analysed after derivatization under five different oxidation conditions: experiment A: mixture under oxygen and without mercapto-ethanol; experiment B: mixture under oxygen with mercaptoethanol; experiment C: mixture under air with mercaptoethanol (i.e. as in ref. 9); experiment D: mixture under nitrogen with mercaptoethanol; and experiment E: mixture under nitrogen with mercaptoethanol and 1—2 mg of sodium dithionite added 3—5 sec before the end of gassing with nitrogen. The dithionite produced some turbidity but this did not interfere with the chromatography.

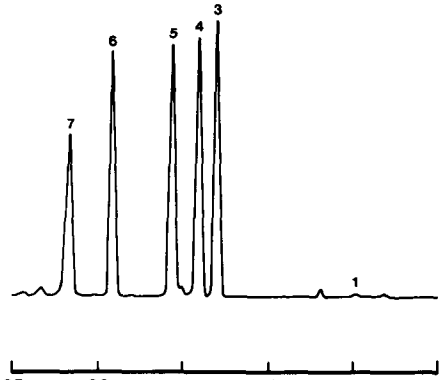
Chromatography

The apparatus comprised a Model 3B pump, an LC-100 oven at 50°C, a Rheodyne 7125 valve injector (100-μl loop) and a Model 3000 fluorescence spectrophotometer fitted with a 16-μl flow cell (all Perkin-Elmer). Excitation and emission wavelengths were set at 322 and 391 nm, respectively. An on-line degasser (Erma Optical Works, Model 3310) was used to reduce possible quenching of fluorescence by oxygen dissolved in the mobile phase. The column (250 mm × 4.6 mm I.D.) was stainless steel packed with 5-μm LiChrosorb RP-8 bonded silica (HPLC Technology). A silica pre-column was used to enrich the mobile phase with silica.

The mobile phase system of Hayashi et al. [9] was used with omission of the ion-pairing reagent and consisted of acetonitrile—water (4:1, solution A) and acetonitrile—water—0.1 *M* sodium dihydrogen phosphate—sodium hydroxide buffer, pH 7.0 (1:12:7, solution B). A linear gradient of 30—80% A was run over 30 min, at a flow-rate of 1.5 ml min^{-1}.

RESULTS AND DISCUSSION

A typical separation of BCKA quinoxalinols, prepared in the absence of oxygen (experiment E) is shown in Fig. 1. This contrasts with Fig. 2 which shows their separation when prepared under oxygen (experiment A) and also shows an almost complete loss of the KIV peak and a considerable increase in the size of the KIC peak. The reason for the increase is not known although the formation of a co-eluting species when oxygen is not excluded, seems possible.



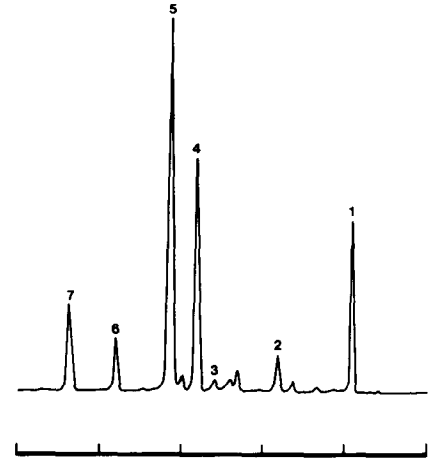


Fig. 1. HPLC chromatogram of the α-keto acids derivatized in the absence of oxygen (experiment E). Peaks: 3 = KIV; 4 = KMV; 5 = KIC; 7 = KOA; 1 and 6 are unidentified.

Fig. 2. HPLC chromatogram of the α-keto acids derivatized in the presence of oxygen (experiment A). Peaks: 3 = KIV; 4 = KMV; 5 = KIC; 7 = KOA; 1, 2 and 6 are unidentified.

The presence of oxygen produced little change in the peak area of the KMV derivative but a reduction in the peak area for the derivative of the internal standard, KOA. Peaks labelled 1 and 2 appeared only when oxygen was present during derivatization. The nature of these components was not investigated but the appearance of peak 1 was a sensitive indication of the presence of oxygen during derivatization of the standard mixture of BCKA (this indication does not neccessarily apply to some biological samples).

The effects of decreasing the extent of the oxidizing conditions on the peak area ratios are shown in Fig. 3. All three BCKA derivatives had similar peak area ratios when prepared under nitrogen and in the presence of mercaptoethanol and sodium dithionite. With increasing oxygenation of the reaction mixture the peak area ratios showed increasing divergence. Variations between peak area ratios from sample to sample were generally smallest in the absence of oxygen. Some of the variability was probably due to the inclusion of varying amounts of oxygen in the different tubes in each experiment since the volume of the Sovirel tubes used for derivatization differed from one tube to another, even though tubes measuring 160 × 16 mm were always used. Gassing with nitrogen alone was not always sufficient to remove all the oxygen as judged by the appearance of a small peak at position 1 in some instances, and divergence of the peak area ratios from closely similar values. A separate experiment had been performed similar to experiment D but with gassing with nitrogen for a prolonged time (10 min, approximately twenty times longer). This served to remove all effective oxygen as indicated by only very small peaks at position 1 on the chromatograms of four replicates. In view of the volatility of the α-keto acids at room temperature under the acidic conditions present during the gassing stage, prolonged gassing would not seem advisable.

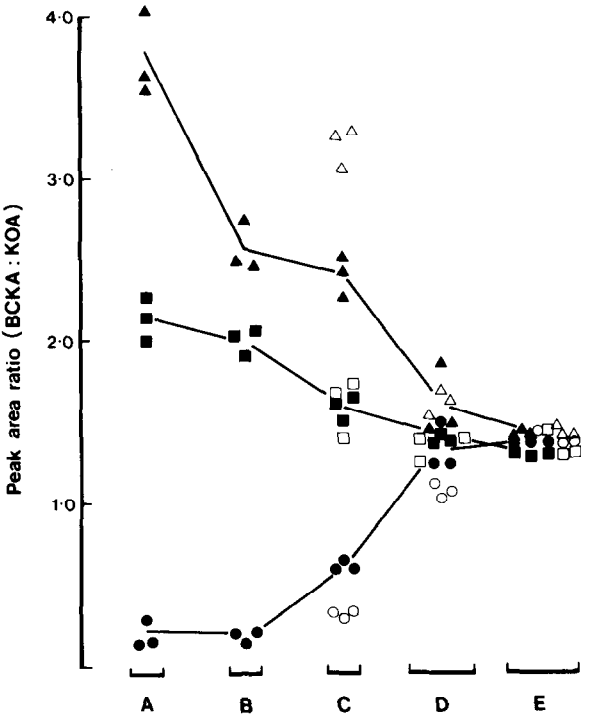


Fig. 3. Effects of progressively decreasing the extent of the oxidizing condition during derivatization on the peak area ratios for BCKA:KOA. Results are for experiments A (most oxidizing) to E (least oxidizing) (see Experimental). Symbols represent: (△, ▲) KIC; (□, ■) KMV; (○, ●) KIV. Closed symbols are for experiments and replicates for which derivatives were prepared simultanenously and open symbols represent experiments conducted on separate days but replicates derivatized simultaneously in each experiment. The lines join points which are the mean values of the data represented by the closed symbols.

Browning of the reaction mixture occurred when oxygen was present during derivatization. A colourless solution did not, however, indicate adequate exclusion of oxygen, since some colourless solutions of derivatives (mostly from experiment D and some from experiment C) showed sizeable peaks at position 1 on the chromatograms, divergence of the peak area ratios from similar values and diminution of the peak area for the KIV derivative, all indicating some effective oxygen had been present. We have no evidence of a mechanism by which oxygen interferes in the derivatization and oxidation of precursors or products would be presumptive.

It is interesting to determine whether the presence of oxygen has interfered with the analysis of BCKA in other published work. We have no indication from the literature of attempts to exclude oxygen from the derivatization mixture although it is possible that mercaptoethanol has been used [9, 10] to reduce auto-oxidation of the *o*-phenylenediamine [18]. The stoppered tube as described by Hayashi et al. [9] for derivatization contains a large air space above the derivatization mixture suggesting oxidation was possible. Some indication of the effect the contained oxygen might have had can be gained by an examination of the pattern of the relative peak area ratios. The peak area

ratio for the KMV derivative is least affected by oxygen during derivatization; if the peak area ratio for this keto-acid is then taken as unity, the relative peak area ratios for the KIC, KMV and KIV derivatives (calculated from ref. 10) are found to be approx. 2.5, 1.0, 0.75, respectively. These values correspond to 1.5, 1.0 and 0.4, respectively in the present study for samples derivatized under air. Rather similar relative peak area ratios are obtained using GC methods when *o*-phenylenediamine is used as the primary derivatizing reagent: from the data of Woolf et al. [8] calculations show relative values of 1.3, 1.0 and 0.8 and from that of Cree et al. [2] relative values of 1.2, 1.0 and 0.7. As KIC and KMV are isomeric, similar responses would be expected, particularly in GC methods where a flame-ionization detector is used. Variations in the relative peak area ratios between different laboratories will reflect differences in instrumentation as well as the extent of interference by oxygen during derivatization. However, the similarity in the pattern of relative peak area ratios suggests that interference by oxygen during the derivatization step may be a common but previously unrecognized problem.

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