Simultaneous Quantitation of Alpha-ketoglutaric Acid and 5-Hydroxymethylfurfural in Plasma by HPLC with UV and Fluorescence Detection

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Alpha-ketoglutaric acid (KG) and hydroxymethylfurfural (HMF) are currently being investigated in clinical trials as an approach in targeted cancer therapy. Hence, a method for the simultaneous determination of KG and HMF in plasma has been developed. Due to the strongly discriminative chemical properties of KG and HMF, SPE purification is performed using an ion-exchange cartridge to separate KG, and a hydrophobic polymeric cartridge to separate HMF. The cartridges are connected together for several steps, thus resulting in a quicker approach for the purification of plasma samples. The derivatization step is based on the reaction of the carbonyl groups of KG and HMF with dansylhydrazine (DNSH) catalyzed by trifluoroacetic acid. The formed derivatives could be separated by reversed-phase LC on a C8-column, and analyzed by UV and fluorescence detection in a single run using a gradient program. The obtained results show good reproducibility, specificity, and detection limits down to the low picomole range.

Keywords Ketoglutaric acid, hydroxymethylfurfural, HPLC, UV and fluorescence detection, SPE

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Introduction

Alpha-ketoglutaric acid (KG) is an endogenous metabolite of the Krebs cycle that has shown great potentialities in the treatment of cancer cells. Beside angiogenesis suppression activity,^{1,2} which has already become a rational reason for the use of this substance in the treatment of many tumours, KG covers important functions in many biochemical pathways. In particular, KG has a pivotal role in aerobic glycolysis. It has been reported that this mechanism appears to be truncated or altered in one or more steps, particularly with regard to the citric acid cycle, and that in a significant number of cancer types anaerobic glycolysis is the main route for energy production.³ This shift in the preferred energy production pathway also leads to an overproduction of acids, such as lactate. Therefore, the administration of KG is intended to restore the normal functionality of the Krebs cycle.

5-Hydroxymethylfurfural (HMF) is a Maillard reaction product widely studied for its uncertain role in the genesis and proliferation of cancer cells, and for its antitumor properties.^{4,5} HMF is supposed to explicate its anticancer action in two ways, either by forming Schiff's base with ammonia and endogenous amines that tumor cells need to avoid the risk of apoptosis due

to the acidic environment created by their growth, or directly damaging those cells through interactions with reacting oxygen species.

KG and HMF are currently being investigated as components of an infusion solution for the treatment of NSCLC (non-small cell lung carcinoma) patients not responding to any conventional treatment.⁶ KG and HMF are also the active compounds of a commercially available preparation used as a perioperative micronutrient supplementation in lung surgery. The first results obtained by in vivo tests and clinical studies⁷ have enhanced the demand for a method capable of detecting them in human plasma.

The quantification of KG can be performed by direct spectrophotometric analysis,⁸ by HPLC coupled to UV detection with^{11–14} or without derivatization^{9,10} and by fluorometric detection.^{15,16}

For the analysis of HMF, various HPLC methods have been developed that often involve reversed-phased techniques, followed by UV detection.^{17,18} Pre-column derivatization approaches are also reported.¹⁹

We already provided a method for the simultaneous determination of KG and HMF with GC-MS,²⁰ and we investigated the behavior of these molecules with regard to HPLC, and thus providing a first method²¹ based on the simultaneous derivatization of KG and HMF with 2,4-dinitrophenylhydrazine. However, further improvements are required, while the present work focuses on the obtained

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achievements.

Providing methods capable to purify and analyze more molecules at the same time is a main focus in bioanalytical chemistry. When dealing with structures as strongly different as KG and HMF, many factors have to be considered, tested, and optimized in order to reach a compromise with respect to sample preparation and the analytical system.

SPE purification is a well-established technology that allows the quick and simple extraction of specific compounds from complex matrixes. Therefore, it has become an elite choice. It could be said that one of the few disadvantages of this technique is the difficulty to separate molecules with different chemical structure with a single procedure. Hence, it would be a great advantage to develop procedures capable of combining different cartridge types in order to achieve a quicker and simpler method that rationalizes the purification of samples, such as plasma or urine. An interesting approach in this direction is the combination of one or more steps of the SPE procedure that should be conducted with the cartridges connected together.

Applying a derivatization step before HPLC analysis is a common procedure to increase the signal intensity of an analyte. Choosing a derivatizing agent able to react simultaneously with different chemical groups is surely an advantage. However, to achieve satisfactory results, the optimized reaction conditions for each molecule must be investigated and balanced together. Both KG and HMF possess a carbonyl function that can be targeted as a reactive group for derivatization with reagents, such as dansylhydrazine (DNSH), a well-known fluorescence labelling reagent that can be monitored under UV detection as well.^{22,23} Since ketones and aldehydes react differently, both reaction parameters, such as time, temperature and the reagent-substrate ratio, as well as chromatographic conditions and detection systems should be carefully investigated and combined together.

Experimental

Reagents and materials

Individual standards of KG and HMF in highest analytical quality ($\geq 99\%$) were kindly provided by C. Y. L. Pharmazeutika GmbH. (Lieboch, Austria). DNSH (Fluka Chemicals, Buchs, Switzerland) was used as received without any other purification. The internal standards, 5-(4-nitrophenyl)furfural (NPF, 98%) and 2-oxovaleric (OV, $\geq 98\%$) acid, were purchased from Sigma-Aldrich (Wien, Austria). Potassium dihydrogen phosphate (KH_2PO_4 , GR for analysis), sulfuric acid (H_2SO_4 , 2 M, analysis grade) and trifluoroacetic acid (TFAA, 99%) were purchased from E. Merck (Darmstadt, Germany). All solvents used were of HPLC grade (Merck). Glucose monohydrated was purchased from Fluka (Vienna, Austria). Bi-distilled water was used for all of the aqueous solutions, unless otherwise noted.

20 mM KH_2PO_4 -buffer was prepared by dissolving an appropriate amount of potassium dihydrogen phosphate in bi-distilled water, and the pH was adjusted to 2.5 using 85% phosphoric acid.

All solutions as well as the mobile phases were filtrated through a 0.45- μm membrane filter.

A stock solution of 1% (w/v) DNSH was freshly prepared by dissolving 100 mg in 10 mL of ethanol while paying attention to the substance not being exposed to direct light source, and by using a dark glass container. A stock solution of 5% (v/v) TFAA was prepared by spiking 250 μL of TFAA in 4.75 mL acetonitrile. Stock solutions of KG and HMF were prepared by transferring a weighted amount of each substance in a volumetric

flask, and dissolving it with bi-distilled water for a final concentration of 5 mg/mL. Standard solutions with different concentrations were prepared by diluting the stock solutions with ethanol. A 0.1% (w/v) stock solution of OV was prepared by spiking 10 μL of 98% OV into a 10-mL volumetric flask, and filling up with ethanol. Additionally, a stock solution of 0.1% (w/v) NPF in acetonitrile was freshly prepared weekly. Due to the insufficient solubility in ethanol, the use of acetonitrile for NPF was preferred. All solutions were stored at 4°C, and further diluted if needed.

To determine recovery rates *via* SPE human plasma, samples were spiked with KG, HMF, and internal standards, and then analyzed by HPLC in combination with both detectors. All samples were freshly prepared prior to use. The concentration of endogenous KG was determined in pooled plasma, and taken into consideration.

HPLC analysis

The HPLC system comprised a Beckman 125/127 solvent delivery module (0 – 29 MPa) and a Beckman 166-UV detector as well as a Shimadzu RF-10A-LX fluorescence detector connected in series in this order. Chromatographic records were obtained using Gold Nouveau software, Version 1.7 (Beckman Technologies). Chromatographic separations were performed on an RP-8-column LiChroCart® 125 – 4 mm equipped with a LiChroCart® RP-18 4 $\times$ 4 mm guard column. Both the column and the guard column were purchased from Merck.

Injections were performed using a manual injector (Rheodyne, Model 7725 i) and an AS-4000-A intelligent autosampler (Merck-Hitachi), both equipped with a 20- μL loop.

Analyses were accomplished by gradient elution with a phosphate buffer (20 mM, pH 2.5) and acetonitrile, with a starting ratio of 70:30 (v/v) and linear progression (within 5 min) to 30:70 (v/v) after a 4-min run. The flow rate was constantly set to 1 mL/min. The monitored UV wavelength was 254 nm, and the excitation and emission wavelengths of the fluorescence detector were 350 and 525 nm, respectively. KG was monitored with UV detection, while HMF was monitored with fluorescence detection.

SPE analysis

SPE analyses were carried out using a 12-position vacuum manifold (Phenomenex, Aschaffenburg, Germany). Different ion-exchange cartridges were tested: SAX, 100 mg/1 mL (Varian, Darmstadt, Germany); Strata SAX, 200 mg/3 mL (Phenomenex); and Strata X-AW, 33 μm Polymeric Weak Anion, 200 mg/3 mL (Phenomenex). Also, different polymeric RP cartridges were tested: Oasis HLB, 30 mg/1 mL (Waters, Vienna, Austria); and Strata X 33 μm polymeric reversed phase, 200 mg/3 mL (Phenomenex).

Table 1 lists the different combinations of solvents used for every step with regard to the different cartridges used.

The recovery rate was assessed by comparing the peak area of spiked plasma samples with standard solutions of both compounds and internal standards. Tests were replicated for each cartridge ($n = 6$) with samples containing 5 $\mu\text{g/mL}$ of each substance.

The optimized procedure was carried out as follows: The Varian SAX column was activated with 3 mL of 1 M HCl. Then, this column (at the top) was connected with the Phenomenex Strata X cartridge, and both were conditioned with 5 mL of ethanol and 5 mL of water; 1 mL of diluted plasma containing KG, HMF and internal standards was used for sampling. Washing ensued with 2 mL of water. After disconnection, the SAX cartridge was eluted with 500 μL of

Table 1 Solvents used for the SPE procedures and recovery rates for KG and HMF

	Ion-exchange cartridge		
	Varian SAX	Phenomenex Strata SAX	Phenomenex Strata X-AW
Activation	3 × 1 mL 1 M HCl	3 × 1 mL 1 M HCl	3 × 1 mL 1 M HCl
Conditioning	5 × 1 mL MeOH 5 × 1 mL H ₂ O	5 × 1 mL H ₂ O	5 × 1 mL MeOH 5 × 1 mL H ₂ O
Sampling ^a	1 mL	1 mL	1 mL
Washing	2 × 1 mL H ₂ O	2 × 1 mL H ₂ O	2 × 1 mL H ₂ O
Elution	500 µL 0.5 M H ₂ SO ₄	500 µL 0.5 M H ₂ SO ₄	500 µL 0.5 M H ₂ SO ₄
Recovery, % KG ^c	104.0 ± 2.9	92.2 ± 2.2	57.8 ± 15.6

	Polymeric RP cartridge	
	Oasis HLB	Phenomenex Strata X
Conditioning/activation	5 × 1 mL MeOH 5 × 1 mL H ₂ O	5 × 1 mL MeOH 1 × 1 mL 1% HAc
Sampling ^{a,b}	1 mL	1 mL
Washing	2 × 1 mL H ₂ O	2 × 1 mL H ₂ O
Elution	300 µL 50% MeOH	300 µL 50% MeOH
Recovery, % HMF ^c	88.2 ± 1.5	90.5 ± 1.9

a. Plasma samples were diluted 1 to 3 with double-distilled water. Final concentration loaded was 5 µg/mL for both KG and HMF.

b. When the system was set as double cartridge, sample amounts were loaded on the first cartridge, the ion-exchange one.

c. $n = 6$.

0.5 M H₂SO₄, and the polymeric cartridge with 300 µL of 50% MeOH into the same vial.

Optimized derivatisation procedure

The SPE eluate was brought to dryness and re-dissolved in 110 µL of ethanol and 10 µL of acetonitrile; 160 µL of DNSH (1% w/v), 100 µL of TFAA (0.75% v/v), and 20 µL of bi-distilled water were added, reaching a final volume of 400 µL. The derivatization was performed at 50°C for 20 min.

For kinetic studies, the reaction tubes were capped and left in a water bath for different time periods at different temperatures between room temperature (RT) and 100°C. The reaction mixtures were then left to cool down for 15 min at RT, and then centrifuged. Finally, 20 µL were directly injected into the system.

Results and Discussion

Optimization of sample pre-cleaning

A first method for the purification of plasma samples was described in our previous work.²¹ To obtain a shorter operational time without compromising the recovery rate and reproducibility already achieved, we investigated the possibility of a double cartridge system for pre-cleaning plasma samples. Our first attempt was realized using a polymeric Oasis HLB and an ion-exchange SAX column. We chose 50% MeOH and 100 mM phosphate buffer (pH 4.5), as washing and elution solvents for KG, respectively, whilst the same solvents were simply inverted for HMF. In these first tests, the only step with the cartridges connected was the sampling. All other steps of the pre-cleaning procedure were performed with the corresponding cartridges

separately. Since KG is normally found in blood, its content in blank plasma samples has to be determined and considered. The results obtained were not satisfactory both from the viewpoint of the reproducibility and the recovery rate, especially with regard to KG. In this first method, the polymeric column was placed above the ion-exchange one, and we found that a great amount of KG was already retained by the polymeric stationary phase.

Since the retention of HMF by the ion-exchange column would be rather improbable, the first procedure variation was the inversion of order between the two columns. This allowed an increase in the recovery rate of KG from 10% up to 50%, but at the same time up to 20% of HMF was found in the washing fraction.

Since the yield of KG was still low, changes on both the washing and the elution solvents were performed. The elution solvent was found to be not strong enough with respect to the pH and consequently the 300 µL of 100 mM phosphate buffer was replaced by 500 µL of 0.5 M H₂SO₄. The modification enhanced the recovery rate of KG up to 75%.

In order to reduce the amount of HMF found in the washing phase of the SAX cartridge, just after the sampling the two units, still connected, were washed with 2 mL bi-distilled water. The reasoning behind these tests was that HMF should show a higher affinity for water than for the ion-exchange stationary phase, and therefore should be released from the upper column, transferred into the lower column, and finally retained there. The results showed that only traces of HMF could be found in the washing fraction of the SAX column, thus confirming our hypothesis. Moreover, the washing step with water made unnecessary any following washing, and in this way reducing the operational time.

With this procedure, different columns were tested. As shown in Table 1, the best results were achieved with the Varian SAX column for KG and with the Phenomenex Strata X for HMF.

At this point, the recovery rate for the internal standards was assayed and recorded as 82.8% for NPF and 86% for OV, thus not necessitating any modification of the optimized procedure.

Optimization of the derivatization reaction

DNSH is a well-known reagent for fluorescence labeling. The reaction is characterized by the formation of an azine intermediate that, after the elimination of water, proceeds to the corresponding hydrazone. The derivatization procedure was investigated from different points of view, since the formation of hydrazones can be influenced by many factors. The reaction pattern for the derivatization of KG and HMF is given in Fig. 1. In order to increase the yield of the reaction and the efficiency of the method, we investigated the chemical aspects and the chromatographic conditions with and without plasma. For quantification we used two different internal standards, OV and NPF that mimic the chemical structure of KG and HMF, respectively, so that any possible differences in the SPE and the reactivity toward the derivatizing agent can be easily overcome.

The percentage of water in the reaction mixture and the pH were the first aspects to be evaluated. Both have already been investigated,²⁴ but their influence on derivative formation may be different for KG and HMF.

An increase in water lead to a strong loss of the derivative up to its complete disappearance with a portion of water equal to 50% of the total volume in the case of the reaction with KG. Similar tests were performed to determine the most suitable amount of TFAA; 5% of water and 25% of 0.75% TFAA were confirmed to be optimal, also for the reaction between DNSH and KG and HMF.

Fig. 1 Reaction of KG (a) and HMF (b) with DNSH (c) to their corresponding hydrazones (d and e).

When optimizing a derivatization reaction, a critical parameter is the ratio between the analytes and the derivatizing agent. Figure 2 shows the relationship between the concentration of DNSH and the formation rate of KG and HMF derivatives. The test was conducted at 50°C for 15 min, with increasing concentration of DNSH. The appropriate amount of the derivatizing agent was found to be different for the ketone and the aldehyde. The curve of KG reaches a plateau when the ratio is 1 to 10, while for HMF the reaction is already maximized at a ratio of 1 to 5. This difference in the concentration requirements was also observed for the internal standards, thus confirming that a ratio of at least 1 to 10 between analytes and derivatizing agents is a prerequisite for the optimal reaction conditions.

Another aspect to be evaluated is the kinetics of formation of the derivatives. To achieve an exhaustive comprehension of the reaction kinetics we observed the formation rate of both the KG and HMF derivatives, while taking the temperature and the reaction time into consideration.

Figure 3 shows the formation rate of KG and HMF derivatives in relation to two different temperatures, 50 and 80°C, and different reaction times of between 1 and 30 min. Reactions have been monitored in triplicates. Most of the published protocols for DNSH derivatisation report 50°C as the reaction temperature.^{23,25} Parallel to this, we tested other temperatures to investigate the possible gain in terms of the reaction time and

Fig. 2 Dependence of KG and HMF derivative formation on increasing concentrations of DNSH. The KG and HMF concentration was 1 μmol/mL.

Fig. 3 Formation rate of KG and HMF derivatives in relationship to time and temperature.

the derivative yield. After a preliminary test with temperatures ranging from room temperature to 100°C, 80°C was chosen owing to its better reaction time/yield ratio (data not shown), and subsequently compared to 50°C. Interestingly, a reaction

only between KG and DNSH at room temperature could be observed.

It appears clear that the temperature has an important role on the yield of the reaction. Although the two curves show the same trends, at 50°C the amount of product is clearly greater than at 80°C.

OV and NPF showed both a close similarity to their corresponding reference compounds, and in comparison they showed a slightly better reactivity. For OV, this is probably due to the absence of the second carboxylic group, whereby for NPF the presence of the nitrophenyl moiety can act as a stabilizer for the intermediate, and therefore could be considered as a catalyzing factor.

Taking into account all of the parameters, the final derivatisation procedure was established as described in the experimental part. As a compromise between the kinetic parameters of the KG and the HMF reaction the temperature was set at 50°C. Although 5 and 10 min seemed to be appropriate, the reaction time was set at 20 min because of the better precision at this time point.

Optimization of the chromatographic conditions

The KG derivative and the HMF derivative possess different chemical characteristic. KG with its two free carboxylic groups needs an acidic mobile phase to be isolated, and the use of a buffer is essential. On the other side, the HMF derivative is a quite hydrophobic molecule, and the use of a great amount of strong buffer can result in a long chromatographic run without any perceptible improvement of separation.

In order to achieve better separation and a suitable chromatographic run time, the use of a gradient elution system is therefore indispensable. The final gradient elution program was established starting from a 70:30 buffer: ACN (phosphate buffer, 20 mM, pH 2.5), which was linearly brought after 4 min to a 30:70 buffer:ACN within 5 min.

Even though fluorescence detection is a powerful detection technique in comparison with UV detection, it can be heavily affected by the water and buffer amounts in the mobile phase. To investigate the loss of signal intensity, we compared the peak areas of KG derivatives generated with UV and fluorescence detection. Increasing the amount of buffer (50, 60, and 70%) in the mobile phase decreases the response of the UV detector minimally, while the fluorescence response, already lower at 50%, suffers a greater loss. Since KG needs an amount of buffer not below 70% in order to obtain a satisfying separation, under these conditions UV detection should be preferred to fluorescence detection. At the same time, HMF is preferably separated under more hydrophobic conditions, and therefore it can be quantified more sensitively by fluorescence detection. The establishment of a HPLC system consisting of a UV detector and a fluorescence detector is the best choice to increase the sensitivity of the method.

The different behaviors of KG and HMF also suggest the use of two different internal standards capable of efficiently replicating the reactions under investigation as well as the behavior both during SPE and HPLC separation. However for analyzing KG and HMF by means of fluorescence detection the application of OV as an internal standard would also be a possibility.

Figure 4 shows chromatograms of a plasma sample spiked with internal standards and HMF using UV and fluorescence detection. KG was not added, since it is an endogenous compound of about 5–10 µg/mL. No interferences with blank plasma were observed. The retention times are 5.9, 12.8, 13.6, and 18.2 min for the dansylated derivatives KG, HMF, OV, and

Fig. 4 Chromatograms of a plasma sample containing the four substances after SPE purification with UV detection (upper chromatogram) and fluorescence detection (lower chromatogram). DNSH (a), KG-DNSH (b), HMF-DNSH (c), OV-DNSH (d), PNF-DNSH (e).

Table 2 Analytical parameters of the method with human plasma samples

Compound	Linearity range ^a	Intra-day precision, %RSD ^b	Inter-day precision, %RSD ^b	LOD ^c	LOQ ^c
KG	1.5–100	4.3	4.7	0.5	1.5–
HMF	0.5–50	3.1	3.0	0.3	0.5
OV	–	2.4	4.1	–	–
NPF	–	2.9	3.9	–	–

a. $R^2 > 0.99$, value are expressed as amount pro-injection volume ng/20 µL. b. Three concentrations were tested ($n = 6$). c. LOD and LOQ are expressed as amount pro-injection volume (ng/20 µL).

PNF.

It can be clearly seen that the response of the fluorescence detector is considerably higher for HMF and OV, while the UV detector assures a better sensitivity for KG and PNF. Against our expectations, reduced fluorescence was observed for the PNF derivative, which may be caused by the nitro group of the molecule.

In order to evaluate the potency of the whole method developed we tested the inter- and intra-day precision as well as the linearity range. The specificity was proved by a comparison with blank reaction and blank plasma samples. A signal-to-noise ratio of 3 and 10 has been used to calculate LOD and LOQ, respectively. As shown in Table 2, the method possesses satisfactory precision and sensitivity, and therefore its use in pharmacokinetic monitoring studies can be considered a suitable choice. In addition to its high sensitivity and high recovery rates, the merit of this method is also the possibility of simultaneous UV and fluorescence detection.

The developed method was compared with our GC/MS technique²⁰ after the infusion of KG and HMF to cancer patients. By this the specificity could be confirmed. Corresponding to the ICH guidelines Q2 (R1), validation of the analytical procedures, the accuracy is given between +4.0% and –9.5% for a concentration of 250 ng/20 µL. The chromatograms of

real-life samples are almost identical to the chromatogram shown in Fig. 4. No interferences of plasma by-products with analytes were observed. The concentrations for KG ranged between 8 and 25 µg/mL, and the concentrations for HMF were at the LOQ level, depending on the metabolism. Detailed data will be shown in a complete pharmacokinetic study.

Conclusions

The need for a reliable method for the simultaneous quantification of HMF and KG in the plasma of cancer patients is required in order to study the pharmacokinetic behavior of these two compounds. Due to the complexity of such real-life samples, the development of a highly selective and sensitive method has been the leading purpose of this work.

The developed SPE procedure can considerably decrease the operational time for the sample preparation, enabling at the same time an adequate recovery. The pre-column derivatization procedure with the use of DNSH can ensure results with LOQ and LOD in the picomole range. It is the first time that DNSH has been used for the derivatization of KG and HMF.

Moreover, the use of both UV and fluorescence detection in series assures the possibility for the simultaneous detection of compounds with such a different chemical structure without compromising neither the sensitivity nor the separation performance. Finally, the method presented can be considered a suitable approach for evaluating of the pharmacokinetic profiles of cancer patients.

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