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A GC/MS validated method for the nanomolar range determination of succinylacetone in amniotic fluid and plasma: An analytical tool for tyrosinemia type I

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Abstract

A sensitive and accurate stable isotope dilution GC/MS assay was developed and validated for the quantification of succinylacetone (SA) in plasma and amniotic fluid (AF). SA is pathognonomic for tyrosinemia type I, a genetic disorder caused by a reduced activity of fumarylacetoacetate hydrolase (FAH). In untreated patients, SA can easily be measured in plasma and urine because the expected concentrations are in the μmol/L range. Due to a founder effect, the province of Quebec has an unusually high prevalence of tyrosinemia type I, hence, the quantification of SA in AF or plasma of treated patients in the nmol/L range becomes very useful. The method utilizes ^{13}C5-SA as an internal standard and a three-step sample treatment consisting of oximation, solvent extraction and TMCS derivatization. The assay was validated by recording the ion intensities of *m*/*z* 620 for SA and *m*/*z* 625 for ISTD in order to demonstrate the precision of measurements, the linearity of the method, limit of quantification and detection (LOQ and LOD), specificity, accuracy, as well as metabolite stability. Values for the intra-day assays ranged from 0.2 to 3.2% while values for the inter-day assays ranged from 1.9 to 5.6% confirming that the method has good precision. A calibration plot using SA detected by GC/MS gave excellent linearity with a correlation coefficient of 0.999 over the injected concentration range of 5–2000 nmol/L. LOQ and LOD were 3 and 1 nmol/L, respectively. The usefulness of this method was demonstrated by SA quantification in an AF sample of an affected fetus and in plasma of patients treated with NTBC. The results demonstrate that this novel GC/MS method may be a valuable tool for metabolic evaluation and clinical use.

Keywords: Tyrosinemia; GC/MS; Succinylacetone; NTBC

1. Introduction

Hereditary tyrosinemia type I (HT1; McKusick 27670) is an autosomal recessive disorder caused by a deficiency in fumarylacetoacetate hydrolase (FAH) in the tyrosine degradation pathway [1] (Fig. 1). This inborn error of metabolism can cause severe liver disease and renal tubular dysfunction [2,3]. Whereas this deficiency is relatively rare in other regions of the world, it is frequently encountered in the province of Quebec due to a founder effect [4]. The FAH deficiency leads to an increase in the release of two toxic molecules: fumarylacetoacetate (FAA) and maleylacetoacetate (MAA) which are readily converted to succinylacetone (SA). This specific biomarker was identified in 1977 in urine of patients with HT1 [5]. Historically, the determination of tyrosine in plasma or on dried blood spots has been the primary biochemical test for the diagnosis of HT1. However, it is now well known that tyrosine levels can increase as a result of dietary changes [6] or a benign transient tyrosinemia [7] in the newborn and thus generate false-positives, thereby rendering tyrosine measurement as a non-specific test for the diagnosis of HT1. Currently, the diagnosis of HT1 relies entirely on the demonstration of increased levels of SA accompanied by a deficiency in FAH. Up until the early eighties, treatment of this disease was solely based on a restricted diet of phenylalanine and tyrosine, followed by a liver transplant as a second alternative. In 1992, a potent inhibitor of 4-hydrophenylpyruvate

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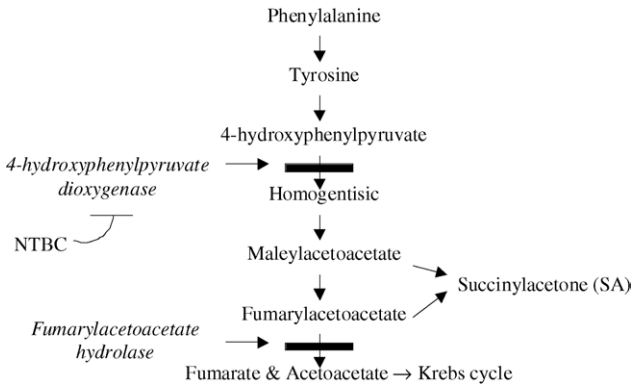

Fig. 1. Pathway of tyrosine degradation and action of the therapeutic agent 2-(2 nitro-4 trifluoromethylbenzoyl)-1,3 cyclohexadione (NTBC).

dioxygenase, namely 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedion (NTBC), was found applicable to HT1 patients [8]. Treatment with NTBC has now become the mainstay for the management of HT1 and provides significant improvement of symptoms in patients [8,9] by drastically reducing the levels of FAA, MAA and SA in biological fluids.

In untreated patients, SA can easily be measured in plasma or urine because expected concentrations are in the μmol/L range. Some well-established methods are used to diagnose the disease, most of which rely either on GC/MS, GC/MS/MS, capillary electrophoresis (CE) or tandem mass spectrometry (MS/MS) for direct quantification of SA [10–17] or are based on inhibition of δ-aminolevulinc acid dehydratase [18]. However, NTBC administration treatment reduces the levels of SA to near the normal range after only a few weeks, hence, the majority of current, published methods do not offer sufficient sensitivity or have not been designed for measuring SA levels in healthy individuals or at the basal range. To our knowledge, there are no reported methods using stable isotope dilution mass spectrometry that have been validated and that have demonstrated the sensitivity and capability of providing a biochemical follow up for HT1 patients under NTBC treatment. Such a method would allow to efficiently monitor the pharmacological response to NTBC by plasma SA analysis prior to and in the subsequent weeks after initiation of therapeutic treatment.

The current report presents a validated isotope dilution mass spectrometry method for measuring SA whilst demonstrating its application in the pre and postnatal diagnosis of HT1 as well, and its importance for monitoring SA in treated patients.

2. Methodology and design

2.1. Chemicals and reagents

Succinylacetone (4-6-dioxoheptanoic acid) and *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) were obtained from Sigma (St. Louis, MO, USA); ^{13}C5-succinylacetone (^{13}C5-SA stable isotope internal standard) from Cambridge Isotope Laboratories (MA, USA): *N*,*O*-bis-trimethylsisyl-trifluoroacetamide (BSTFA) from Regis Technologies, Morton Grove (IL, USA) and chromatographic grade ethyl acetate and diethyl ether from Fisher Scientific (Ont., Canada).

Stock solutions of SA (125 μmol/L) and ^{13}C5-SA (30 mmol/L) were prepared in water and stored at −80 °C. Working solutions (50 and 100 nmol/L, respectively) were obtained from stock solutions by dilution, kept at −80 °C and discarded after 1 month.

2.2. Sample extraction and derivatization

Fresh SA standard solutions (5, 25 and 50 nmol/L) were prepared daily by appropriate dilution of the working solution. To 1 mL of standard, amniotic fluid (AF) or 0.5 mL of plasma (brought up to 1 mL with deionized water), 50 μL of ^{13}C5-SA (100 nmol/L solution) internal standard was added plus 40 μl of H_2SO_4 0.25 mol/L and 100 μL PFBHA (50 mg/mL) in order to perform oximation for 60 min at room temperature (RT) [19,20]. Thereafter, pH was adjusted to 1 by adding 40 μL of 5 mol/L HCL to each tube saturated with 0.1 g of sodium chloride. The acidified sample was successively extracted with twice 2 mL of ethyl acetate and twice 2 mL of diethyl ether with vigorous shaking. The organic layers were combined into a second tube and the solvent was evaporated to dryness under a gentle stream of nitrogen. The residue was then derivatized with 100 μL of BSTFA and heated for 60 min at 70 °C [21]. Four microlitres of each sample were injected into the GC/MS system.

2.3. GC/MS analysis

An agilent GC/MS system was used consisting of a 6890A model gas chromatograph and a 5973 model inert mass selective detector. A fused-silica capillary column coated with 5% phenyl–95% dimethylpolysiloxane (ZB-5, Phenomenex, 30 m × 0.25 mm i.d., 0.25 mm film thickness) was also used. The GC temperature program was as follows: initial temperature was 80 °C, held for 1 min, increased to 280 °C at a rate of 17 °C/min, held for 3 min. A splitless injection mode was used and 4 μL was injected at 250 °C, transfer line temperature at 270 °C and ion source temperature at 200 °C. The mass spectrometer was operated at 70 eV in the electron impact mode with selected ion monitoring (SIM). The selected ion groups for identification of SA and ^{13}C5-SA in SIM mode were *m*/*z* 620 and *m*/*z* 625, respectively. Dwell time for each ion was set at 100 ms.

2.4. Method validation

2.4.1. Molecule stability

The GC/MS method was validated for the quantification of SA by first investigating molecule stability. Thirty nmol/L SA samples were stored at room temperature 4, −20 and −80 °C over an 8-week period allowing sufficient time to monitor stability. Samples were analyzed immediately after preparation (time zero), with SA determination performed every week for the selected period.