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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.
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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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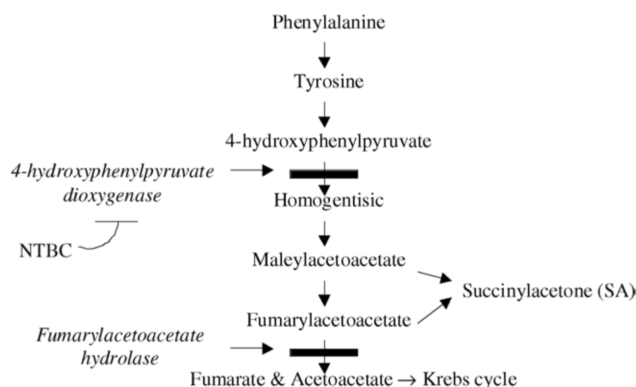

Fig. 1. Pathway of tyrosine degradation and action of the therapeutic agent 2-(2 nitro-4 trifluoromethylbenzoyl)-1,3 cyclohexadione (NTBC).

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.

2.4.2. Recovery and assay precision

The recovery of SA was determined by assaying AF samples spiked with known concentrations of SA (5, 25 and 50 nmol/L). Accuracy assays were performed by evaluating the average coefficient of recovery (%), (experimental value/true value) × 100. Precision was evaluated both intra- and inter-day by analysis of an AF sample fortified with known concentrations of SA (5, 25 and 50 nmol/L) and the data compared after three consecutive runs (intra-day) and from data obtained over a 3-day period (inter-day). Hence, the calculated %CV for both intra- and inter-day assays provides a reliable estimate for the reproducibility and intermediate precision of the method.

2.4.3. Linearity, limit of detection and quantification

Linearity was verified by analyzing standard samples in the 5–2000 nmol/L concentration range. The limit of detection (LOD) was calculated as the concentration producing a peak with a signal-to-noise ratio of at least 3:1 and the limit of quantification (LOQ) was calculated as the minimum concentration added to a blank sample that can be measured within 20% of the target concentration.

2.5. Samples

Two AF specimens from at risk pregnancies for HT1 and positive for SA were performed at 17 and 29 weeks of gestation and were provided by the Quebec Blood Neonatal Screening Program, Laval University Medical Center, Que., Canada. Thirty anonymised AF samples (13–18 weeks of gestation) obtained in our service of medical genetics served as controls. Plasma from HT1 patients pre- and under NTBC treatment was received in our laboratory for amino acid analysis as part of a follow-up procedure for these patients. For controls, thirty anonymised plasma samples from patients referred for biochemical metabolic investigation in our laboratory were used and in which no abnormalities were found.

3. Results and discussion

3.1. Sample treatments and chromatography

In mass spectrometry, the use of compensating standards is essential for maintaining precision and accuracy. The stable isotope dilution technique is the method of choice and refers to the use of a stable isotope-substituted analogue of analyte as an internal standard, added to the sample at the very beginning of the analytical method [22]. To prevent the possible exchange of deuterium and hydrogen atoms between SA and deuterated-SA in the acid medium during the oximation step, the commercially available ^{13}C5-SA was chosen as our stable internal standard isotope. Since it can be added at the very outset of sample preparation, the assay thus becomes a true isotope dilution mass spectrometry reference method. There are currently no published techniques for the measurement of SA in plasma or AF in the nanomolar range that conform to these criteria. Keto acids, such as succinylacetone, which contain an α-hydrogen atom on a carbon adjacent to the keto group, are subject to keto-enol tautomerism and therefore, in order to protect α-ketoacids in preparation, the carbonyl group must be converted to oxime-, methoxime- or ethoxime-derivatives prior to extraction [23,24]. In the present study, PFBHA was used to oximate SA, since this reagent offers high molecule stability. Moreover, no intramolecular reaction can occur during oximation, in contrast to a likelihood of it occurring if hydroxylamine is used [10]. Very few methods have been used to extract SA from biological fluids prior to GC/MS analysis: one method requires a relatively long time span, while necessitating lyophilization and a liquid partition chromatography technique [13], whereas the others are based on liquid–liquid extraction [12,23,25]. Solvent extraction, on the other hand, is simple and we were able to confirm the efficiency of this known method [10,26] by comparing, in a triplicate assay, the peak areas of SA standards obtained after solvent extraction with those obtained after evaporation of pure oximated standards prepared in methanol (unextracted standards). The mean efficiency of extraction compared to the unextracted sample was 76% (data not shown). Following extraction, SA was derivatized by trimethylsilylation (TMS), this method being useful in the analysis of volatile organic acids by making them less volatile, more thermally stable and suitable for GC/MS analysis. As a result, GC separation is improved and detection is enhanced. Two different silylation reagents were used, namely BSTFA and BSTFA + 10% trimethylchlorosilane (TMCS), where BSTFA alone provided a slight improvement in overall signal-to-noise ratio.

Fig. 2 presents a typical chromatogram of SIM analysis of a standard sample. An HT1 plasma sample compared to a normal plasma sample, as well as an HT1 patient under NTBC treatment for the past 3 months are shown in Fig. 3. SA was converted into four different derivatives when treated with PFBHA. Following trimethylsilylation, the four isomers corresponding to two *cis* and two *trans* chemical configurations were separated by capillary GC yielding a specific chromatographic pattern that improved assay specificity. In addition to targeting the molecular weight [M + H] *m/z* 620 for SA, this chromatographic profile must be present to confirm molecular identity. In

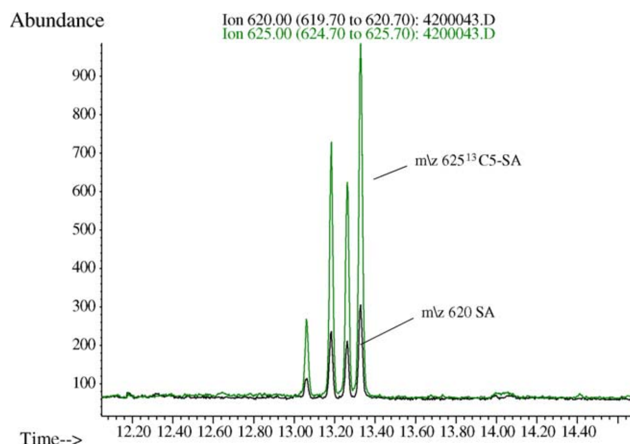

Fig. 2. GC/MS ion chromatogram of SIM analysis of four isomers of the SA derivatives (standard: 25 nmol/L, *m/z* 620) and their stable isotope analogue ^{13}C5-SA (100 nmol/L, *m/z* 625).

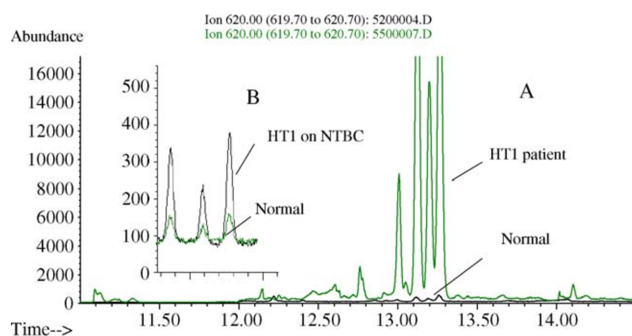

Fig. 3. Comparative GC/MS chromatograms (SIM) of (A) an enlarged chromatogram portion of a normal plasma SA (12 nmol/L) compared to SA from an HT1 patient treated with NTBC for the past 3 months (37 nmol/L) and (B) an HT1 patient at the start of NTBC treatment (2 days, 4.8 μmol/L) compared with an healthy individual (12 nmol/L).

order to establish the standard curve and sample quantifications, the ion abundance of the three predominant isomers were used. At low concentrations, the first isomer with a retention time at 13.05 min, corresponding to the less favorable geometric isomerism, was nearly undetectable. Thus, only the three major isomer peaks of SA and ^{13}C5-SA were considered for quantification.

Fig. 4 illustrates the mass spectra of SA dioxime tri-TMS obtained by GC/MS (scan analysis). The mass spectrum is characterized by the *m/z* 620 ion (molecular ion) and by ions 181 and 73, which are assigned to PFBHA and TMCS, respectively.

3.2. Method validation

Because it is often necessary to store samples for an extended period of time prior to analysis or to provide advice for shipment storage, the evaluation of the stability of SA was therefore determined. Several investigators have previously reported that SA is unstable [23,27], correlating with our results. The stability of SA was evaluated under four different conditions (room temperature, 4, −20 and −80 °C) over an 8-week period. Aqueous standard samples were analyzed immediately after preparation (reference values) and after storage. Our results suggest that SA must be kept at −80 °C in order to maintain complete integrity for storage for 8 weeks. At 4 and −20 °C, mean recoveries were 70% after a 3-week storage period and remained stable for the rest of the study. At RT, SA was drastically degraded resulting in only 18% recovery after 8 weeks of storage.

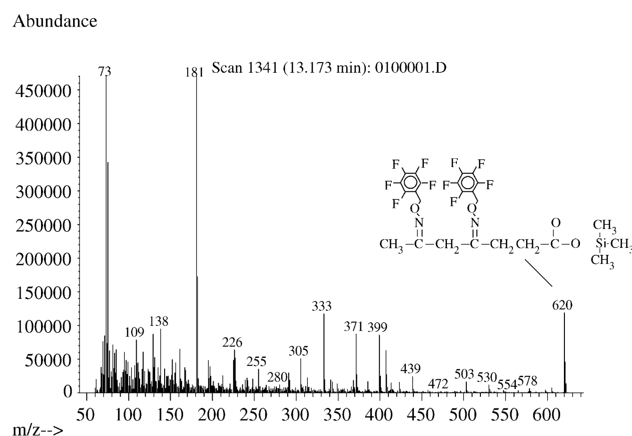

Fig. 4. Mass spectra of succinylacetone dioxime-TMS.

Table 1
Precision and accuracy of the assay in amniotic fluid

Amount added (nmol/L)	Mean ($n = 3$) measured concentration (nmol/L)	Mean recovery (%)	Precision ($n = 3$)	
			Intra-day (%CV)	Inter-day (%CV)
0	4.6	NA[a]	NA	NA
5	9.3	97.2	3.2	5.6
25	29.1	98.5	0.2	2.3
50	50.4	92.4	1.7	1.9

[a] NA, not applicable.

The mean overall coefficient of SA recovery (%) from an AF spiked with 5, 25 and 50 nmol/L of SA using the liquid–liquid extraction and recalculated with a standard curve established with the same procedure was 95.7% (see Table 1) which was well within the 85–115% range normally used as a criterion in method validation. In the current method, LOD and LOQ were 1 and 3 nmol/L, respectively. To verify these parameters, 5 nmol/L SA was used as the lowest calibration point on every analytical run as well as verify that the signal-to-noise ratio was at least five times greater than any interference.

A calibration plot using SA detected by GC/MS gave excellent linearity with a correlation coefficient of 0.999 for the three major isomer peaks over the injected concentration range of 5–2000 nmol/L. Intra-day and inter-day precision of mass spectrometry detection were evaluated by analyzing control samples spiked with SA (5, 25 and 50 nmol/L) (see results in Table 1).

In addition to accuracy, precision was used to assess the suitability of the method whereby the calculated coefficient of variation (%CV) values should not exceed 15%. 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.

3.3. Clinical applications

The present method was applied to determine both AF and plasma concentrations of SA for the purpose of prenatal diagnosis, as well as the pharmacological response in the follow up of an HT1 patient under NTBC treatment, respectively. To date, this novel method has been successfully applied to the analysis of over 300 AF and 60 plasma samples where statistical data are shown in Table 2. The sensitivity needed to achieve an unambiguous prenatal diagnosis exceeded several orders of magnitude in this study and is demonstrated in Fig. 5. Initially, the present method was developed to perform a HT1 prenatal test in our biochemical genetics laboratory, but its high sensitivity and specificity enabled to further extend this method to the study of HT1 disease and its treatment with NTBC. HT1 is a disease frequently encountered in the province of Quebec, for which there are no published data providing SA plasma concentrations in order to monitor HT1 patients, prior to NTBC treatment

Table 2
Plasma and amniotic fluid SA concentration in the control group and HT1 patients

Samples	SA concentration (range, S.D.) (nmol/L)
Amniotic fluid control ($n = 30$)[a]	Mean = 3 (0–13, 3)
Affected case 1	2102
Affected case 2	2012
Plasmas control ($n = 30$)[b]	Mean = 13 (3–21, 3.6)
HT1 treated patients ($n = 21$)[c]	Mean = 33 (21–55, 8)

[a] 13–17 weeks of gestation.
[b] All ages.
[c] All patients are treated from at least 7.5 months, age average = 7 years, range = 0.7–32 years.

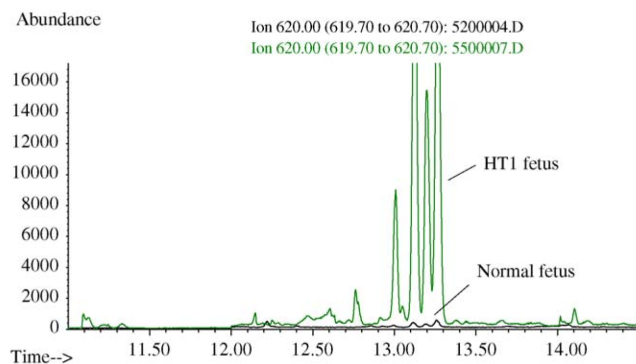

Fig. 5. Chromatogram obtained from amniotic fluid of an HT1 fetus (2012 nmol/L) compared to a normal (4 nmol/L) fetus. The four SA isomers are shown (m/z 620). For better chromatographic clarity, the m/z 625 from ^{13}C5-SA was not extracted.

(at time of diagnosis) and several weeks thereafter, until the concentration reaches a plateau at about 6 S.D. above normal. Also, during this study, we periodically received plasma samples from a newly diagnosed HT1 patient through the Quebec Blood Neonatal Screening Program. The samples were received 2 days following initiation of treatment. Fig. 6 provides a pharmacological response curve established for this patient using the assay method described herein.

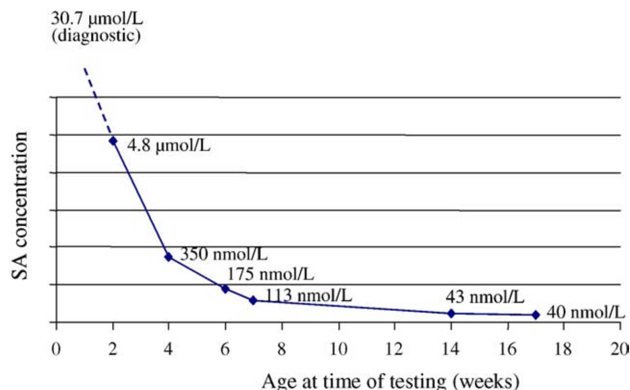

Fig. 6. SA profile of plasma concentrations over in an HT1 patient following initiation of NTBC treatment.

4. Conclusion

The method developed herein is both useful and reliable not only for prenatal diagnosis and pharmacological response studies but can also be applied as a confirmatory test for the indirect quantification of SA based on inhibition of δ-aminolevulinate dehydratase used in mass screening. The signal-to-noise ratio and the robustness of the quantification have been enhanced by a new analyzer equipped with an ionization source coated with an inert material and a turbo molecular pump enabling to raise the vacuum. The most important advantages of the present GC/MS for AF and plasma SA are its relative simplicity in sample preparation, the fact that it is a validated method respecting all criteria related to a stable isotope dilution procedure, its capability to monitor the decrease in plasma SA following NTBC introduction, to values nearing the basal range and finally, that it requires less sophisticated and less expensive instrumentation compared with the use of tandem mass spectrometry.

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