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Journal of Chromatography B, 831 (2006) 274–280
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of succinylacetone in dried blood spots and liquid urine as a dansylhydrazone by liquid chromatography tandem mass spectrometry

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Received 12 July 2005; accepted 8 December 2005
Available online 27 December 2005

Abstract

Succinylacetone (SA) is a specific marker for the inherited metabolic disease, hepatorenal tyrosinemia. We developed a stable-isotope dilution liquid chromatography tandem mass spectrometry for the determination of SA in dried blood spots (DBS) and liquid urine using a $^{13}C_4$-SA as internal standard. SA was extracted, converted to the butyl ester and derivatized with dansylhydrazine (Dns-H). Calibration curves in DBS and urine calibrators were linear up to 100 and 30 μM, respectively. At a signal-to-noise ratio of 3, the limits of detection in DBS and urine were 0.2 and 0.005 μM, respectively. Total run time was 5 min. Intra- and inter-assay precision expressed as coefficient of variation were better than 9.1% with more than 96% recovery. The method was applied retrospectively and prospectively for the diagnosis of hepatorenal tyrosinemia and for follow-up of patients under treatment.

Keywords: Hepatorenal tyrosinemia; Tyrosine; Succinylacetone; Dansylhydrazine; Tandem mass spectrometry; Newborn screening

1. Introduction

Hepatorenal tyrosinemia (tyrosinemia type-I; HT1) is a rare autosomal recessive metabolic disease caused by a deficiency of fumarylacetoacetate hydrolase (FAH), an enzyme that catalyzes the terminal step in tyrosine (tyr) degradation [1–6]. Toxic metabolites upstream of the enzyme block, namely, fumarylacetoacetate (FAA) and maleylacetoacetate are converted into succinylacetoacetate (SAA) which decarboxylates to give succinylacetone (SA; 4,6-dioxoheptanoic acid), which is elevated in body fluids of HT1 patients. It is believed that these toxic agents are the cause of the extensive clinical, pathological and neurological manifestations leading to liver cirrhosis, renal failure, hypophosphataemic rickets, hepatocellular carcinoma and death in early childhood [1,7,8]. HT1 is considered a treatable metabolic disease provided it is detected early in life and treated with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC; Nitisinone^{TM}), the only alternative available for liver transplantation [9].

Biochemical findings associated with HT1 such as elevated α1-fetoprotein, hepatic transaminases, tyr, methionine (met), delta-aminolevulinic acid, 4-hydroxyphenylpyruvate, 4-hydroxyphenyllactate and 4-hydroxyphenylacetate are non-specific markers and are elevated in a number of other liver diseases in childhood [3,4]. SA is not known to occur as an intermediate in any other metabolic pathway; therefore it is considered a specific diagnostic marker for HT1. Determination of FAH activity in human liver, lymphocytes and fibroblasts is restricted to patients with elevated SA due to the large number of cases presenting with non-specific clinical and biochemical findings [10,11].

SA in plasma, urine and amniotic fluid was determined by gas chromatography–mass spectrometry (GC–MS) [12–16]. Traditionally, urine was the preferred matrix for diagnosis and treat-

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1570-0232/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2005.12.010

ment follow up for HT1 cases. This is due to the polarity of SA that has a relatively high renal clearance where the levels are about three times higher in urine than in plasma [14]. Recently, we described a liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS–MS) for the analysis of SA in urine [17]. This method involved the use of ^{15}N-labeled 5(3)-methyl-3(5)-isoxazole propionic acid as internal standard. Urine samples from patients suspected with HT1 were converted to the isoxazole derivative by hydroxylamine HCl, extracted from the matrix followed by derivatization to the butyl ester. Although the method was sufficiently sensitive for urine, it was not sensitive enough to allow us to determine SA in dried blood spots (DBS).

The introduction of MS–MS-based methods for newborn and selective screening through the analysis of blood spots for amino acids (AAs) and acylcarnitines (ACs) for a multitude of inherited disorders made the DBS specimens the most frequently received sample in biochemical genetics laboratories due to the convenience of blood transport on filter paper plus the fact that it can be used readily for both biochemical and molecular testing [18]. Actually, in our previous report most of the 12 new HT1 cases were suspected and later confirmed due to the finding of high met (197–864 μM; cut-off 63 μM) and high tyr (207–681 μM; cut-off 200 μM) in DBS specimens by MS–MS analysis [17].

In the literature, there are two reports describing the analysis of SA in DBS. Schulze et al. reported a second-tier spectrophotometric method for the indirect determination of SA in DBS based on inhibition of delta-aminolevulinate dehydratase enzyme (ALA-D) by SA [19]. Allard et al. presented a primary screening method for HT1 by ESI–MS–MS analysis of SA from blood spots that were already extracted for AAs and ACs analysis [20].

In the work presented here we sought to develop a sensitive approach for the determination of SA that would allow us to reach a definitive diagnosis for clinically or biochemically suspected HT1 cases from the same DBS specimens that are used for AAs and ACs determination. This approach may eventually eliminate the need for urine analysis.

2. Experimental

2.1. Chemicals

($^{13}C_4$)Succinic anhydride (99 at.%) was purchased from Isotec. 4,6-Dioxoheptanoic acid (SA), thionyl chloride, *t*-butylacetoacetate were obtained from Sigma–Aldrich. 5-Dimethylaminonaphthalene-1-sulfonyl hydrazine (dansylhydrazine, Dns-H) was purchased from Fluka Chemie. Trifluoroacetic acid (TFA), methanol, HPLC grade acetonitrile, HPLC grade ethylacetate and ammonium acetate were obtained from Fisher. Butanolic HCl was from Regis Technologies. Water used throughout this study was prepared by reverse osmosis and further purified by passing through a Milli-Q System (Millipore, Bedford, MA, USA). Filter paper (S&S 903) used for blood spot collection was purchased from Schleicher and Schuell (Dassel, Germany).

2.2. Synthesis of (1,2,3,4-$^{13}C_4$) 4,6-dioxoheptanoic acid

The synthesis of the labelled SA internal standard (IS) was accomplished by a described procedure with slight modifications [21]. The ($^{13}C_4$) succinate mono methyl ester was prepared by refluxing ($^{13}C_4$) succinic anhydride (0.52 g) and dry methanol (0.18 g) in carbon tetrachloride (15 ml) for 16 h. The solvent was evaporated, and the mono-ester was mixed with thionyl chloride (0.97 g) and warmed in an oil bath at 50 °C for 3 h. After cooling, excess thionyl chloride was removed in a stream of nitrogen till constant weight. The residue was dissolved in dry ether (5 ml) and slowly added to a stirred suspension of the magnesium complex of *t*-butyl acetoacetate (1.06 g) in ether (50 ml). The mixture was refluxed for 2 h, cooled to ambient temperature, acidified with 0.1 N HCl (20 ml), and stirred for 10 min. The ether layer was separated, and the aqueous layer was extracted with ethyl acetate (15 ml). The combined organic fractions were washed with water, dried over $MgSO_4$, filtered and evaporated, yielding the crude di-ester as an off-white solid. This di-ester was taken up in formic acid (10 ml), and decarboxylated by heating at reflux for 3 h. The solvent was evaporated, and the crude product was purified over silica with chloroform yielding methyl ($^{13}C_4$) 4,6-dioxoheptanoate (0.41 g) as a slightly yellow liquid. Thin layer chromatography of a sample showed one single spot (R_F 0.35, $CHCl_3$). This ester was hydrolysed in refluxing 3 N HCl (7 ml) for 2 h. The product was isolated by extraction with methylene chloride and recrystallised from ether/hexane, yielding 0.31 g of ($^{13}C_4$) SA as an off-white solid. The chemical purity and isotopic purity of $^{13}C_4$-SA was checked by ESI–MS and further analyzed in the derivatized form by LC–MS–MS (see below).

2.3. Control and patients specimens

For the determination of SA reference range we used DBS specimens received for newborn and selective screening ($n = 500$) and urine from patients ($n = 139$) received for organic acids analysis. Only specimens reported as "unremarkable" were used. In addition, we analyzed the first DBS specimens for 13 HT1 patients that were received for AAs and ACs analysis and three were for follow up for the same analytes from patients undergoing NTBC treatment. These samples were stored at room temperature under dry conditions for up to 30 months. Urine samples for SA determination from HT1 patients ($n = 11$) stored at −20 °C for up to 30 months were employed.

2.4. Sample pretreatment and derivatization conditions

To a single 3/16″ disc punched out from DBS specimens in a 13 mm × 100 mm screw-cap borosilicate glass tube, aliquot of 0.5 ml of 0.5 M HCl containing the IS (1.5 μM) was added. The tubes were tightly capped and heated at 100 °C for 30 min. This step is taken to convert any SAA to SA and to release SA from any adducts [15]. After cooling to room temperature, excess sodium chloride and 1 ml of ethylacetate were added successively. The mixtures were vortex-mixed for 2 min and centrifuged at 3800 rpm for 5 min. After collecting and evaporating the organic layer under a stream of N_2 at 40 °C, 50 μl

of butanolic HCl were added and the mixtures were incubated at 65 °C for 15 min followed by evaporation to dryness under N_2 gas at 40 °C. The residues were reconstituted in 100 μl of 2.5 mM of Dns-H in acetonitrile and 10 μl TFA (2.5%, v/v) in acetonitrile and allowed to stand at ambient temperature for 1 h in the dark. Ten microliters were subjected to the analysis.

To analyze urine samples, 100 μl of urine were placed in a test tube and 10 μl of IS (20 μM) and 20 μl of HCl (5 M) were added. This sample was treated exactly as described above for DBS samples. Urine specimens with SA levels outside the linear range of the assay were diluted with water (4–20-fold), re-extracted and re-analyzed.

2.5. Liquid chromatography and mass spectrometry systems

An Agilent 1100 HPLC system composed of a binary HPLC pump, autosampler and on-line degasser was employed. Separation was performed on a C8 column (150 × 2.1 mm i.d., 5 μm, Symmetry, Waters) preceded by a guard column (Symmetry C8, 20 × 2.1 mm i.d., 3.5 μm, Waters). A 78% acetonitrile containing 2 mM ammonium acetate at a flow rate of 0.3 ml/min was used as a mobile phase. All column effluent was directed into an Applied Biosystems API 2000 bench-top triple quadrupole mass spectrometer equipped with a Turbo Spray ion source and a built-in syringe pump. Mass calibration and resolution adjustment on the resolving quadrupoles were performed automatically by continuous infusion of a polypropylene glycol solution. The peak width at half height was set on both resolving quadrupoles at 0.75 atomic mass units. The system was operated with source temperature of 400 °C, a needle potential of 5500 V, a declustering potential of 18 V and collision energy of 30 eV with nitrogen as a collision gas. Analyst software version 1.4 was used to control the MS system and for data handling.

2.6. Method validation

Stock solutions of SA were prepared by dissolving the proper amount in water to give a concentration of 10 mM. These solutions were stored at 4 °C in the dark and were stable for at least one month. To determine the linear range, DBS calibrators were prepared by adding SA stock solution to control whole blood to yield 100 μM, which was further diluted with control blood to give 75, 50, 25, 10, 2.5, and 1 μM. Spiked blood calibrators were applied manually onto S&S 903 filter papers and allowed to dry at room temperature overnight. Dried calibrators were stored under dry conditions at −20 °C with desiccant.

For urine, calibration curves were constructed using pooled control urine with a creatinine value of 2 mM spiked with SA at the following concentrations: 0.025, 0.05, 0.1, 0.5, 1, 5, 10, 20, and 30 μM. These samples were stored at −20 °C except during use.

The recovery, intra- and inter-day variations of SA determination was assessed by repeatedly analyzing DBS calibrators (2.5 and 50 μM) and spiked urine (0.1, 1 and 20 μM) as described earlier over a period of 10 days. Repeatability was evaluated by repeatedly injecting the same sample containing SA at 2.5 μM onto the LC–MS–MS ($n = 6$). Method accuracy was further evaluated by analyzing urine specimens from known HT1 cases before ($n = 11$) and after NTBC treatment ($n = 5$), together with four control specimens by the proposed method and simultaneously by our previously published method [17].

3. Results and discussion

3.1. Internal standard

To the best of our knowledge, this is the first report on the synthesis of stable-isotope $^{13}C_4$-SA internal standard. This isotope is more stable compared to its deuterium antipode which may undergo exchange during sample preparation which involves heating in acidic medium and has advantages over our previously reported ^{15}N-labeled 5(3)-methyl-3(5)isoxazole propionic acid used as IS in previous reports [14,15,17]. The main advantage is that the $^{13}C_4$-SA differs by 4 mass units from SA and it showed no signal in the SA channel and vice versa. This simplified the calculation by eliminating the need for corrections for peak areas obtained for each analyte, which was absolutely necessary for the ^{15}N-labeled IS. A $^{13}C_5$-SA labeled standard that would serve the same purpose became commercially available in 2004 from Cambridge Isotopes Laboratories, Inc.

3.2. Method development

SA is a di-oxo aliphatic acid with very poor ionization efficiency in both negative and positive ion ESI–MS. In this work, we were concerned with the preparation of a SA derivative with high ionization efficiency to allow us to measure SA in DBS specimens. For this purpose we attempted the use of Dns-H, a reagent known as an excellent fluorescence label for carbonyl compounds in HPLC that yields relatively stable hydrazones [22]. Dns-H contains a dimethylaminonaphthyl moiety and therefore was expected to improve ionization efficiency of the derivative.

Both positive and negative ion ESI–MS and ESI–MS–MS were used to analyze the products of the reactions of SA and IS (data not shown). Despite the use of excess of Dns-H, we found that the reaction proceeded to form a product that corresponds to the mono-dansylhydrazone derivatives of SA and IS. No ions corresponding to bis-dansylhydrazone derivatives were observed. LC–MS–MS analysis of this Dns-derivative showed unfavorable chromatographic properties such as co-elution with excess reagent, short retention time and peak tailing. This is presumably due to the amphoteric nature of the derivative, which has both a tertiary amine and a free carboxylic group. Therefore, we converted the Dns-derivative to the butyl ester. The butylated Dns-derivatives of SA (Dns-SA) and its IS (Dns-IS) were analyzed by positive ion ESI–MS and ESI–MS–MS. Two abundant ions at m/z 462 and m/z 466 appeared in the spectra, which correspond to $(M + H)^+$ for Dns-SA and Dns-IS, respectively. Under optimized positive ion collision activated dissociation (CAD) conditions, the product ion spectra revealed a major fragment at m/z 170 common to both derivatives and was assigned to the dimethylaminonaphthyl moiety originated from Dns-H. Among other less abundant fragments the Dns-SA gave frag-

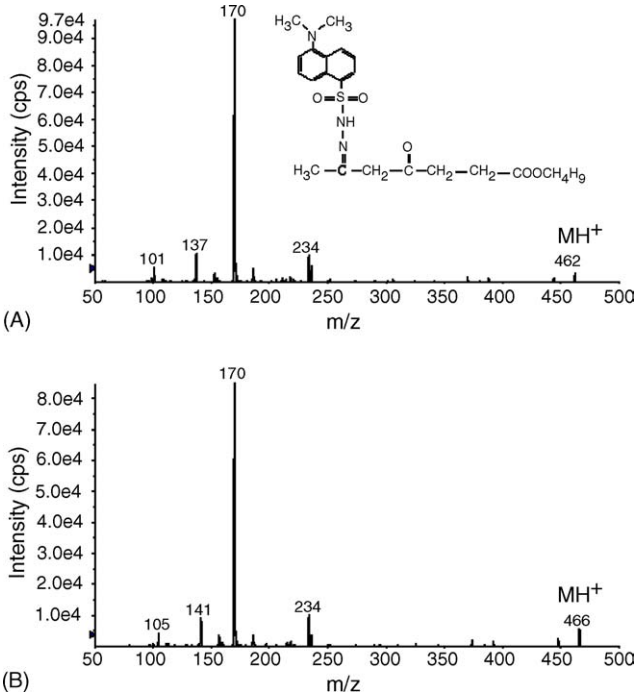

Fig. 1. ESI–MS–MS CAD spectra of *m/z* 462 for Dns-SA derivative (A) and *m/z* 466 for $^{13}C_4$-labeled Dns-IS derivative (B). Detailed conditions are described in experimental section.

ments at *m/z* 101 and 137 and Dns-IS gave the corresponding fragments at *m/z* 105 and 141 carrying the $^{13}C_4$ skeleton with abundance of 10–15% relative to that of common fragment at *m/z* 170 (Fig. 1). The butylated Dns-SA and Dns-IS derivatives gave more favorable ionization efficiency as compared to the unbutylated dansyl derivatives, and were retained longer on a reversed-phase column, which allowed us to use a higher organic modifier concentration in the mobile phase with further improvement in the ionization process.

The MS–MS results described above were used to develop an LC–MS–MS method. One selected reaction monitoring (SRM) scan function was programmed to include the following transitions: *m/z* 462 → 170 and *m/z* 462 → 137 for SA and *m/z* 466 → 170 and *m/z* 466 → 141 for the IS. The fragmentation of the precursor ions to the common fragment at *m/z* 170 were used for quantification and the fragmentation to the less abundant fragments were used for confirmation resulting in greater sensitivity with confidence regarding the compound identity. Dns-SA and Dns-IS derivatives co-eluted with a retention time of 2.5 min, while excess Dns-H reagent eluted earlier at 1.5 min. A complete run time of 5 min was employed to ensure the elution of a late peak that appeared in the SA channel. It is noteworthy to mention that the Dns-IS when analyzed separately showed no contribution to signal in the Dns-SA channel. Fig. 2A shows typical chromatograms obtained for HT1 patient's DBS and DBS sample of a patient under NTBC treatment. Fig. 2B shows typical chromatograms obtained for HT1 patient's urine and urine of a patient under NTBC treatment. No interferences were observed at the retention times of the analytes in either DBS or urine specimens and no carryover was observed. The data in Fig. 2 shows clearly the presence of other early or late eluting peaks in the SA

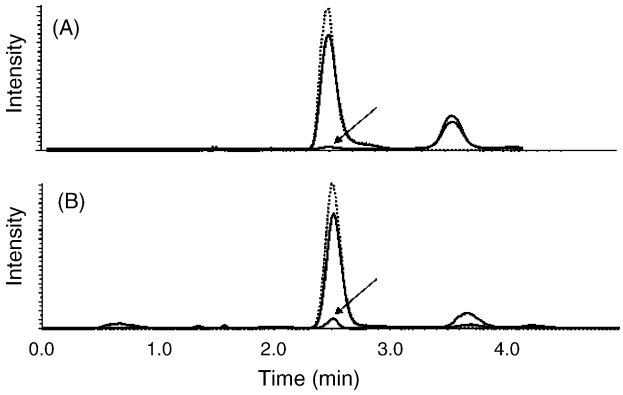

Fig. 2. Positive ion mass chromatograms of SA (*m/z* 462 → 170; solid line) and IS (*m/z* 466 → 170; dotted line) derivatives: (A) Three overlaid chromatograms: SA in DBS of HT1 patient before treatment (57 μM), under NTBC treatment (0.72 μM), and IS; (B) three overlaid chromatograms: SA in diluted urine sample (20-fold) from a HT1 patient before treatment (concentration was 1.6 μM), after NTBC treatment (0.16 μM) and for IS. The arrows are pointing at SA profiles after treatment.

channel from both the DBS and urine, which necessitated the chromatographic step. However, our experience with the current method and our previously reported LC–MS–MS method [17] indicates that profiles obtained here are much less "noisy" particularly at the very low physiological levels of SA in control and NTBC-treated specimens. This is presumably due to the higher molecular mass of the derivative and an enhanced specificity of the MRM transitions.

3.3. Method validation

Calibration curves constructed using DBS calibrators were linear in the range of 1–100 μM. The average (standard deviation) of the slope and *y*-axis intercept of four regression curves obtained on 4 different days were 0.0134 (0.0009) and 0.026 (0.019), respectively, with a correlation coefficient (*r*) of ≥0.996. Calibration curves obtained using spiked urine were linear between 0.025 and 30 μM, a range that covers the normal and many of the abnormal samples concentrations. The average (standard deviation) of the slope and *y*-axis intercept ($n = 6$) were 0.59 (0.01) and 0.019 (0.009), respectively, with a correlation coefficient (*r*) of ≥0.999. The intrinsic selectivity of tandem MS and the sensitivity attained in this work allowed the quantitative determination of SA in a single 3/16″ DBS disc as well as from a small volumes of urine (0.1 ml). The limit of detection in DBS and urine samples at a signal-to-noise ratio (S/N) of 3 were 0.2 and 0.005 μM, respectively. The limit of quantification at S/N of 9 were 0.6 and 0.015 μM, respectively.

Precision expressed as coefficient of variation (CV%) was evaluated using DBS calibrators containing SA at 2.5 and 50 μM and pooled urine spiked at 0.1, 1, and 20 μM to represent low and high concentrations. As shown in Table 1, the highest CV% in DBS and in urine were 9.09 and 7.96%, respectively. This variation is acceptable taking in consideration the multi-step pretreatment procedure and the nature of the biological matrix plus the inherent variability of DBS specimens with regards to

Table 1
Recovery, intra- and inter-day reproducibility of SA in DBS and urine

Sample	SA added (μM)	Within-day				Between-day			
		Mean (μM)	CV[a] (%)	Recovery[b] (%)	n[c]	Mean (μM)	CV (%)	Recovery (%)	n
DBS	2.5	2.54	9.09	101.4	20	2.75	8.35	110.1	10
	50	50.90	8.99	101.9	20	49.26	6.19	98.5	10
Urine	0.1	0.099	4.34	99.1	6	0.099	7.96	98.5	6
	1	0.967	4.44	96.7	6	1.01	6.07	100.9	6
	20	19.25	1.82	96.3	6	19.67	2.15	98.3	6

[a] CV: coefficient of variation.
[b] Recovery (%) = 100 × found concentration/added concentration.
[c] Number of replicates.

filter paper thickness and the degree of saturation with blood. The overall analytical recovery of SA from DBS was calculated from these samples and was in the range of 98.5–110.1% and in urine was 96.3–100.9%. The method repeatability was determined by calculating the CV of concentration determined by repeatedly injecting the same sample within the same day (n = 6) and was 0.82%.

For quality control purposes, we set the accepted interval by calculation (mean ± 2 standard deviation) from results obtained by repeatedly analyzing DBS at 2.5 and 50 μM on different days (n = 30) as 2.1–3.1 and 42.1–58.7 μM, respectively. Quality control samples were included in each batch analyzed and results were accepted if quality control samples fall within the accepted range.

3.4. Analysis of controls and patients samples

The method described here is superior to our previously reported LC–MS–MS method [17]. A 20-fold higher S/N was obtained when the same amount of SA was analyzed as Dns-SA compared to the isoxazole propionic acid derivative. The control range of SA was obtained by the current method using DBS specimens from healthy infants (n = 500). SA was not detectable in 65% of these samples, hence, the control range was between 0 and 0.98 μM with a 99th percentile value of 0.75 μM. In urine from healthy infants (n = 139), the SA ranged between 0.002 and 0.155 μmol/mmol creatinine. This range is in excellent agreement with that previously determined in urine by GC–MS and LC–MS–MS [14,17].

We retrospectively and prospectively applied the current method to the analysis of DBS samples from confirmed or suspected HT1 patients. Table 2 shows the concentration of SA in DBS originally received for MS–MS analysis for AAs and ACs, the diagnosis for 12 of these patients was previously reached [17]. Two new HT1 cases (#14 and #15) were identified prospectively by the current method. They were two-month-old twins that showed signs of abdominal distention and elevated transaminases. MS–MS analysis of DBS for amino acids revealed elevated met in both (361 and 355 μM, respectively) and elevated tyr (216 and 238 μM, respectively). These results together with the clinical symptoms led to the suspicion of HT1.

SA concentration in DBS ranged from 3.3 to 65 μM, a range that is readily distinguishable from the controls by the current method and is similar to values reported earlier by MS–MS and by spectrophotometric analysis [19,20]. In sera, enzymatically determined SA was reported at levels as low as 2 μM and as high as 100 μM [12]. In DBS specimens of HT1 patients receiving NTBC, SA concentrations were not significantly different from the normal range as shown in Table 2.

Concentrations in urine covering control and abnormal SA levels obtained by the current method were checked against results obtained by analyzing the same samples by the isoxazole method. As shown in Fig. 3, there was an excellent agreement between results of the two methods. Results obtained from single determination for each method were analyzed by linear regression. A slope of 1.05 and a correlation coefficient of 0.997 were obtained.

3.5. Methods comparison and diagnostic utility

The methods of direct SA quantification in DBS described here and that of Allard et al. [20] have clear advantages over the indirect semi-quantitative SA determination method of Schulze et al. In the latter method false-positive results may occur due to hereditary ALA-D deficiency, exposure to lead and high temperature [19]. It was also found that EDTA blood showed significant decrease of ALA-D activity. The methods of direct SA deter-

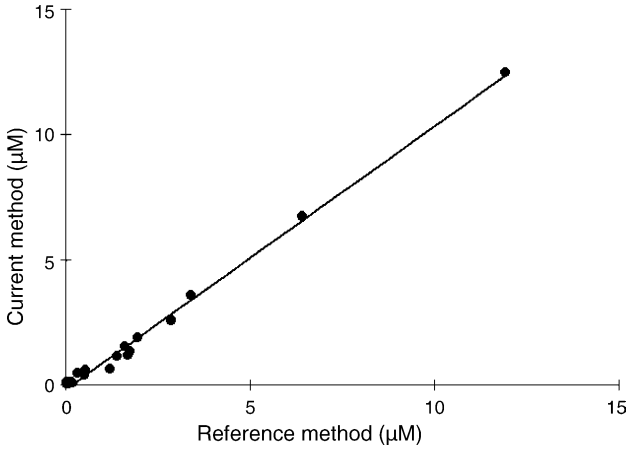

Fig. 3. Linear regression analysis of results obtained by current method vs. a reference LC–MS–MS method for urine specimens (n = 20). Appropriate dilutions of abnormal urine specimens were made to bring the concentration within the linear range of the assay.

Table 2
SA concentration in DBS and excretion values in urine of HT1 patients and controls

Patient number[a]	Age of DBS specimen (weeks)	SA in DBS (after NTBC) (μM)	SA in urine (μmol/mmol creatinine)
1	112	57 (0.8)[b]	40
2	100	32	440
3	36	65	521
4	–	N/A[c]	140
5	80	4.6	111
6	112	19	184
7	92	18	72
8	–	N/A	171
9	44	11	147
10	52	23	29
11	96	15	302
12	72	34 (0.6)[d]	233
13	12	3.3 (1.0)[e]	15
14	0.6	22	196
15	0.6	29	156
Controls		Range = 0–0.98[f]	Range = 0.006–0.14

[a] Urine excretion values for patient 1–12 are from [17].
[b] Sample in parenthesis was collected 60 days after initiation of NTBC.
[c] N/A: no DBS available.
[d] Sample in parenthesis was collected 127 days after initiation of NTBC.
[e] Sample in parenthesis was collected 15 days after initiation of NTBC.
[f] DBS controls ($n = 500$); 99th percentile = 0.75 μM.

mination have no such limitations. In making the comparison between the two direct SA methods our sample pretreatment time may appear more demanding, yet it actually takes 3 h to prepare a batch of 50 samples. Our method has a longer analytical time of 5 min versus 2 min. However, it has a better analyte recovery, lower detection and lower quantification limits as evidenced by the ability of detecting control SA levels in 35% of all DBS specimens tested versus only 0.5% in DBS specimens tested by Allard et al. method [20]. This allowed us to determine a 99th percentile value for SA in control sample to be 0.75 μM rather than setting an arbitrary number of 1 μM. The diagnostic power of our method in DBS was validated retrospectively and prospectively against urine SA determination by two different methods in a relatively large number of HT1 patients ($n = 13$). In this regard, there was a total agreement between the DBS data and the urine data which allows us to claim 100% sensitivity and specificity of the DBS method despite the relatively old age of the DBS specimens and their storage at room temperature. These results further indicate that SA in DBS is relatively stable and that this type of sample can be used to make a diagnosis from samples up to 3 years post-specimen collection.

Allard et al. presented their method as a primary screening method for HT1, which may be particularly useful in high-risk populations such Quebec, Canada where the regional incidence is 1:1846 [1]. We however, do not believe that primary screening is cost-effective for other populations where the incidence is estimated at 1:100,000 [19]. Another approach would be to use the direct SA determination as a second-tier test in newborns found with high tyr and/or met in the routine MS–MS method for AAs and ACs. However, even this approach may not be cost-effective for several reasons. First, in many newborn screening programs DBS specimens are collected at 24 h after birth when the levels of tyr and met may not have exceeded the cut-off levels leading to false-negative results for HT1 cases. Second, elevated tyr due to transient hypertyrosinemia of the newborn, other types of tyrosinemia or other liver conditions may lead to a heavy and unnecessary burden of second-tier testing, medical costs and parents anxiety [1,23,24]. In Quebec, where newborn screening for HT1 has been in effect since 1970, a tyr cut-off value of 414 μM at a mean age of screening of 4 days resulted in 1% recall rate. In 1980s adjustment of the cut-off to 248 μM resulted in 2.4% of newborns tested for SA with a second-tier test [1]. In the state of Georgia, USA a cut-off value of 331 μM at a mean age of sampling of 2 days resulted in a very high recall rate (10%), while a threshold value of 663 μM reduced the recall rate to 0.1% [1]. Furthermore, Schulze et al. estimated a 500 second-tier test for each true positive even based on a high tyr cut-off value of 384 μM which corresponds to 99.5th percentile [19]. In using any of these cut-off values for selective screening we would have missed several of the cases shown in Table 2. Third, HT1 patients have shown excellent response to NTBC treatment if started after few months of life and up to 2 years of age. Consequently, we propose that direct SA determination methods in DBS be reserved for high-risk infants from HT1 affected families, for testing patients with hepatosplenomegaly, porphyria-like symptoms, Fanconi syndrome, rickets, for patients with abnormal liver enzymes with high tyr and/or met and for follow up and control of patients on NTBC of treatment.

4. Conclusion

We report a new approach to the determination of SA, the only reliable and accepted hallmark of HT1. This LC–MS–MS method allows for determination of SA in DBS as well as in

urine specimens with a short analytical time. Based on our retrospective and prospective experiments the analysis of SA in DBS can clearly distinguish between normal and affected infants. The method is convenient that it allows for reaching a definitive diagnosis from the same DBS used for patient screening and may be used for follow-up of treatment, and with further validation may replace the need for urine analysis. DBS analysis may also be useful for high-risk as well as retrospective screening for HT1 from stored DBS.

Acknowledgment

This study was funded by a grant from Prince Salman Center for Disability Research. The authors are grateful for Gulf Bioanalytical Co. for providing the LC–MS–MS system employed.

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