assocation is independent of the traditional markers of response in multivariate analysis, including ER concentrations. In addition to its predictive value for tamoxifen resistance in recurrent breast cancer, as shown here, the prognostic value of BCAR1 in primary breast cancer has been confirmed separately (L.C.J. Dorssers et al., manuscript submitted). BCAR1/p130Cas has also been associated with malignant melanoma and some types of leukemia (12). Recently, overexpression of BCAR1/p130Cas in vitro was shown to interfere with KAI1/CDB2-mediated suppression of metastasis in prostate cancer cells (15). Further evaluation of the role of BCAR1 in various kinds of malignancies and other diseases may benefit from our quantitative detection methodology for BCAR1 protein.

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commercially available, we studied the 3MGH activity in the reverse direction, using HMG-CoA as a substrate. We quantified the formation of 3-methylglutaconyl-CoA by reversed-phase HPLC with ultraviolet detection. The described assay allows the precise measurement of residual 3MGH activity in cultured human skin fibroblasts.

We obtained KH$_2$PO$_4$, H$_3$PO$_4$, Tris, EDTA, HCl, KOH, MES, and acetonitrile (chromatography grade) from Merck. Bovine serum albumin (essentially fatty acid free), KH$_2$PO$_4$, H$_3$PO$_4$, Tris, EDTA, HCl, KOH, MES, and acetonitrile (chromatography grade) from Merck. Bovine serum albumin (essentially fatty acid free), HMG-CoA, and bicinchoninic acid were purchased from Sigma.

We obtained skin fibroblasts from 2 individuals with confirmed 3MGA type I (1, 6), 5 individuals with confirmed Barth syndrome (13), 2 individuals with 3MGA type IV (14), and 13 control individuals with no evidence of an inborn error of branched chain amino acid oxidation or mitochondrial fatty acid oxidation. All cells were initially obtained during the process of diagnosing inborn errors of metabolism.

Fibroblasts were grown and harvested as described elsewhere (15). For use in determining the intra assay (within-day) and inter assay (between-day) variation, we harvested six cell cultures from three control individuals, pooled them, divided them into 20 pellets, and stored them frozen at $-80^\circ$C.

Cell pellets were suspended in 200 g/L of phosphate-buffered saline by repeated pipetting and were sonicated three times on ice for 15 s at 8 W at 45-s intervals. We determined the protein concentration by the bicinchoninic acid assay (Sigma), using bovine serum albumin as the calibrator. The assay mixture contained, in a final volume of 100 g/L, 100 mmol/L Tris (pH 8.0), 10 mmol/L EDTA, 1 g/L bovine serum albumin, 100 mmol/L HMG-CoA, and 10–50 mg/L fibroblast protein. After incubation at 37°C for 60 min, the reaction was terminated by addition of 10 g/L of 2 mol/L HCl. The samples were homogenized, and the assay tubes were placed on ice. After 5 min, the homogenates were brought to pH 6 with 2 mol/L KOH–1 mol/L MES (pH 6) and centrifuged at 21,000g for 10 min at 4°C. Finally, 100 g/L of the supernatant was transferred to a HPLC vial.

For gradient elution, we used a binary solvent system. Solvent A was 100 mmol/L KH$_2$PO$_4$ adjusted to pH 4 with 100 mmol/L H$_3$PO$_4$, filtered before use through a 0.45 μm nitrocellulose membrane under reduced pressure. Solvent B was 200 mL/L acetonitrile–800 mL/L solvent A. Both solvents were degassed for 15 min in an ultrasonic bath (Branson 3510). We injected 50 g/L of sample, and the acyl-CoA esters were eluted at a flow rate of 1 mL/min by a 15-min linear gradient of 20% B to 100% B. Peak areas of interest were integrated by use of Chromelon software package (Dionex).

We first developed a HPLC-based method to allow baseline separation between HMG-CoA and 3-methylglutaconyl-CoA. Separation of acyl-CoA esters by reversed-phase HPLC is dependent on the salt concentration and the pH of the eluent (16). We found that a linear gradient of acetonitrile in potassium phosphate buffer (100 mmol/L, pH 4.0) allowed baseline separation between substrate and product.

Typical chromatograms for incubations with fibroblast extracts from a control individual and a patient with 3MGA type I are shown in Fig. 1B. Using acyl-CoA

![Diagram](image-url)
the substrate and the product were equally hydrolyzed to homogenates of 3MGH-deficient fibroblasts with these HMG-CoA and 3-methylglutaconyl-CoA by incubating reaction mixture are susceptible to enzymatic and chemical hydrolysis. We measured the rate of hydrolysis for HMG-CoA and 3-methylglutaconyl-CoA by incubating homogenates of 3MGH-deficient fibroblasts with these acyl-CoA esters. Under the assay conditions used, both the substrate and the product were equally hydrolyzed to the extent of ~25%. We therefore corrected for hydrolysis by calculating the 3MGH activity as the ratio of product to the sum of product and substrate. However, because HMG-CoA is also a substrate for other enzymes, calculating the 3MGH activity in such manner would give an overestimation of the 3MGH activity.

HMG-CoA could first be converted into mevalonic acid by the action of HMG-CoA reductase (EC1.1.1.34) with NADPH as a coenzyme. However, because NADPH was not added, the action of the reductase on HMG-CoA under the assay conditions was negligible. In addition, preliminary studies revealed that, under the incubation conditions described above, <5% of HMG-CoA was converted into acetyl-CoA by the action of HMG-CoA lyase (results not shown). For correct calculation of the 3MGH activity, the contribution of acetyl-CoA has to be added to the total amount of the CoA esters in the incubation. To summarize, the 3MGH activity was calculated according to the following equation, which expresses the 3MGH activity as nmol/min/mg protein:

$$3\text{MGH} = \frac{\epsilon_1 \times (A) \times \text{input}}{\left[\epsilon_1 \times (A) + \epsilon_2 \times (B + C)\right] \times \text{time} \times \text{protein}}$$

where $A$ is the peak area for 3-methylglutaconyl-CoA; $B$ is the peak area for HMG-CoA; $C$ is the peak area for acetyl-CoA; the molar absorptivities $\epsilon_1$ and $\epsilon_2$ are 22.6 cm$^2$/μmol and 16.0 cm$^2$/μmol (18), respectively; the input is 10 nmol; time is 60 min; and protein is expressed in mg.

The activity of 3MGH in human fibroblast extracts showed little variation as a function of pH in the range 7.0–9.0 (data not shown) as reported by others using the coupled enzyme assay (19). For the standard assay, we selected a pH of 8.0.

The assay was linear with an incubation time up to at least 60 min with 50 mg/L fibroblast protein per assay (data not shown). For the standard assay, an incubation time of 60 min and a protein content of 10–50 mg/L fibroblast protein per assay was chosen.

Using optimized assay conditions, we reinvestigated fibroblasts from two patients with established 3MGH type I (1, 6) and detected no formation of 3-methylglutaconyl-CoA in either case. Inclusion of 10 mL/L control homogenate in a homogenate from a patient with 3MGH type I, corresponding to a 3MGH activity of 20 pmol/min/mg protein, could readily be detected. Hence, the 3MGH activity in both patients was <20 pmol/min/mg protein.

The intraassay variation, estimated by assaying 10 of the pooled control pellets in a single experiment, was 4.0% [mean (SD) = 2.26 (0.09) pmol/min/mg protein]. The interassay variation was 4.8% [mean (SD) = 2.2 (0.1) pmol/min/mg protein; n = 10 days].

Measurement of 3MGH activity in 13 control fibroblast homogenates revealed a mean (SD) 3MGH activity of 2.1 (0.7) pmol/min/mg protein [range, 1.0–3.6 pmol/min/mg protein]. The mean (SD) 3MGH activity in fibroblasts from five Barth syndrome patients was 2.8 (0.8) pmol/min/mg protein [range, 2.2–4.2 pmol/min/mg protein]. The two patients with confirmed 3MGA type IV had 3MGH activities of 1.59 and 1.02 pmol/min/mg protein, respectively. These values for 3MGH activity were within the range for controls. Hence, the 3MGH activity in cultured fibroblast homogenates from patients with Barth syndrome or 3MGA type IV appears to be within reference values. This corresponds with the finding that 3MGH activity was normal in patients with Barth syndrome and 3MGA type IV by the coupled enzyme assay (3).

In conclusion, we present a sensitive and specific enzymatic assay for 3MGH that enables the rapid enzymatic diagnosis of 3MGA type I in cultured human skin fibroblasts without the need for radiochemicals. Baseline separation between the substrate HMG-CoA and the product 3-methylglutaconyl-CoA was achieved. Our novel procedure allows detection of 1% residual 3MGH activity in patients with 3MGA type I. To date, all of our patients with 3MGA type I demonstrated a 3MGH activity below this limit. Our method could be useful for studies of genotype/phenotype correlation as well as for studies of the enzymatic characterization of mutated proteins produced in *Escherichia coli.*

References

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Automated Turbidimetric Benzalkonium Chloride Method for Measurement of Protein in Urine and Cerebrospinal Fluid, Fatma Meric Yilmaz, Nermín Celebi, and Dogan Yuceel (Ministry of Health, Ankara Hospital, Clinical Biochemistry Laboratory, Ankara 06340, Turkey; * author for correspondence: fax 90-312-3621857, e-mail doyucel@yahoo.com)

Various methods have been used to measure protein in urine and cerebrospinal fluid (CSF). Urinary protein can be determined by turbidimetric assays using trichloroacetic acid (1), sulfosalicylic acid (2), or benzethonium chloride (BTC) (3, 4); the biuret assay (5, 6); or protein dye-binding assays using Coomassie Brilliant Blue (CBB) (7–13), Pyrogallol Red (PYR)-molybdate (14, 15), or Ponceau S (16). The BTC, CBB, and PYR methods can be automated. Shephard and Whiting (17) used benzalkonium chloride (BC) as a precipitating agent for the determination of protein in urine and CSF by nephelometry. In this study we developed an automated turbidimetric method using BC and compared it with conventional automated methods.

Albumin fraction V (bovine), disodium molybdate · 2 H₂O, NaOH, sodium azide, sodium benzoate, potassium oxalate, and succinic acid were obtained from Merck; BTC, Coomassie Brilliant Blue G 250, EDTA, and PYR were from Sigma; BC was from Serva; and phosphoric acid was from Riedel de Haen. A commercially available PYR reagent (M-TP) and protein calibrators were obtained from Beckman Coulter. A globulin solution containing mostly IgG and IgM and no albumin was obtained from the Refik Saydam Center of Hygiene, Turkey.

We developed a new method that uses the same principle as the method of Iwata and Nishikaze (3) but with BC instead of BTC. BC is a detergent and causes protein denaturation in an alkaline medium. The NaOH/EDTA reagent (reagent 1) contained 33 mmol/L EDTA and 0.5 mol/L NaOH. The BC solution (5 g/L; reagent 2) was prepared from a 50 g/L aqueous stock solution of BC. Calibrators containing 50, 100, 200, 500, and 1000 mg/L protein were prepared from a Beckman serum calibrator (Multical). In a manual procedure, we mixed 2.0 mL of NaOH/EDTA with 200 μL of sample and immediately added 0.5 mL of BC reagent. After a 10-min incubation, we measured the absorbance at 450 nm. We then adapted our method to a Synchron® LX 20 Pro analyzer, using the following conditions: reagent 1 volume, 200 μL; reagent 2 volume, 50 μL; sample volume, 20 μL; primary wavelength, 470 nm; secondary wavelength, 700 nm; blank reading (sample blank), 48–72; reaction time, 10 min; reaction reading, 700–748; calibrators, 50, 100, 200, 500, and 1000 mg/L.

We compared the performance characteristics of our method with those of the Bradford CBB assay (7), the PYR assay (14), and the BTC assay (3). We adapted the PYR and BTC methods to the same analyzer and used the Beckman Coulter M-TP assay, which is also based on the PYR-molybdate method, for comparison. It has previously been demonstrated that the PYR-molybdate method is analytically and clinically comparable to the reference biuret method (15). We measured the total protein concentrations in 100 urine and 88 CSF samples simultaneously by each of the methods. Urine samples were assayed on the day of collection after centrifugation, and CSF samples were stored at −20 °C before analysis. Deming regression analysis, the Pearson correlation coefficient between the methods, and all other statistical calculations were performed with the Microsoft Excel Analyze-it program. The study was approved by the Local Ethics Committee of our hospital.

Samples containing 100-5000 mg/L protein were prepared by adding known amounts of protein calibrators to a urine pool. The PYR assay was linear up to 3000 mg/L; the BC, BTC, and M-TP assays were linear up to 1500 mg/L; and the CBB assay was linear up to 1000 mg/L. Serial dilutions of a sample containing 200 mg/L protein were prepared (range, 6.25–200 mg/L), and a protein concentration that produced an absorbance +3 SD above that for a zero calibrator (n = 21 replicates) was accepted as the detection limit of the method. The detection limits...