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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SERUM SHORT-CHAIN FATTY ACIDS BY DIRECT DERIVATIZATION

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SUMMARY

The application of direct derivatization in conjunction with high-performance liquid chromatography is described for the analysis of short-chain fatty acids in serum. The method is based on the reaction of these acids with acidic 2-nitrophenylhydrazine hydrochloride, without complicated isolation steps, which produces their non-volatile hydrazine derivatives. The hydrazides of fourteen saturated and unsaturated, straight and branched, short-chain fatty acids were separated from other acid hydrazides and interfering components by a simple solvent extraction, and were eluted isocratically on a reversed-phase C_8 column within 24 min. UV detection demonstrated that the detection limits for the acids were 200–400 fmol per injection with linearity over the range from 400 fmol to 5 nmol per injection. Analytical recoveries ranged from 96.8% to 103.1% and coefficients of variation ranged from 0.9% to 3.8%. The present method is simple, accurate and adequate for the analysis of short-chain fatty acids in biological fluids and tissues of patients suffering from organic acidemias.

INTRODUCTION

It is well known that several inborn errors of branched-chain amino acid metabolism are characterized by excessive amounts of various short-chain fatty acids (SCFA) in blood and/or urine [1–8]. The quantitative analysis of SCFA in biological fluids is therefore useful in the diagnosis and management of these metabolic disorders.

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) can handle only volatile substances, and therefore GC-based methods have become a popular means for the analysis of SCFA. Reviews on the use of GC or GC–MS techniques in the investigation of various metabolic diseases have been published by Jellum [9] and by Niwa [10]. GC-based methods commonly involve lengthy and cumbersome isolation steps, such as vacuum distillation, steam distillation, partition chromatography on silica and solvent extraction.

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These procedures may result in loss of sample or in hydrolysis of the endogeneous SCFA esters. Separation of the SCFA by GC-based methods, especially direct injection, may be limited by tailing of the peaks and absorption with appearance of ghost peaks after repeated injections.

In contrast, high-performance liquid chromatography (HPLC) theoretically offers the advantage of simultaneous analysis of volatile and non-volatile substances, but the lack of chromophoric groups in the SCFA molecules makes difficult the detection of the acids that occur in biological materials. Chemical derivatization in HPLC has been the method of choice to increase the sensitivity and selectivity of the detection of fatty acids [11–18]. Therefore, the most notable method for the HPLC analysis of SCFA in biological samples is the direct conversion of the acids into their non-volatile, chromophoric compounds without complicated steps to isolate the acids. However, none of the HPLC methods published have given attention to this problem and also allowed the simultaneous separation of saturated and unsaturated, straight and branched, SCFA.

We have recently developed the utility of the reagent 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) for the derivatization of both short- and long-chain fatty acids, and their separation and quantitation by HPLC with UV–visible detection [19].

This paper reports an improvement of the method for the direct derivatization of serum SCFA and for the HPLC analysis of thirteen clinically important SCFA, including unsaturated and branched acids, using 2-ethylbutyric acid as internal standard.

EXPERIMENTAL

Reagents and chemicals

Lactic, acetic, propionic, *n*-caproic and iso-caproic acids were purchased from Katayama Kagaku Kogyo (Osaka, Japan). *n*-Butyric, iso-butyric, DL-2-methylbutyric, 2-ethylbutyric, *n*-valeric, iso-valeric, crotonic, 3-methylcrotonic and tiglic acids, pyridine and 2-NPH·HCl were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1-EDC·HCl) was purchased from Sigma (St. Louis, MO, U.S.A.). Analytical-reagent-grade methanol and acetonitrile were purchased from Wako (Osaka, Japan). All reagents and chemicals were used without further purification.

Reagent solutions

Aqueous 2-NPH·HCl solution (20 m*M*) and acidic 2-NPH·HCl solution (20 m*M*) were prepared by dissolving the reagent in water and 100 m*M* hydrochloric acid–ethanol (1:1, v/v), respectively. Solutions of pyridine (3%, v/v) in ethanol and of 1-EDC·HCl (250 m*M*) in ethanol were prepared, then a working 1-EDC·HCl solution was prepared by mixing equal volumes of the 1-EDC·HCl and pyridine solutions. A solution of potassium hydroxide (15%, w/v) in methanol–water (4:1, v/v) was prepared.

Derivatization procedure

This procedure has been described previously [19–22]. Briefly, to 100 μl of an aqueous mixture of standard SCFA, 200 μl of aqueous 2-NPH·HCl solution and 400 μl of working 1-EDC·HCl solution were added and the mixture was heated at 60°C for 20 min. After the addition of 100 μl of potassium hydroxide solution, the mixture was further heated at 60°C for 15 min, then cooled in running water.

Assay of SCFA in human serum

To 100 μl of human serum, 10 μl of water containing 1 nmol of 2-ethylbutyric acid as internal standard were added. The serum sample was treated according to the derivatization procedure with acidic 2-NPH·HCl. The resulting mixture of hydrazides was neutralized by addition of 4 ml of 1/30 *M* phosphate buffer (pH 6.4)–0.5 *M* hydrochloric acid (3.8:0.4, v/v) and was washed with 5 ml of *n*-hexane to remove the long-chain fatty acid hydrazides and interfering substances. A 3-ml aliquot of the aqueous layer was taken. The SCFA hydrazides were extracted with 4 ml of diethyl ether. The ether layer was washed with 3 ml of 1/30 *M* phosphate buffer (pH 6.4) and evaporated with a stream of nitrogen at room temperature. The residue was redissolved in 50 μl of methanol, and an aliquot (5–10 μl) was injected into the chromatograph.

HPLC analysis

A Shimadzu LC-5A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with a Shimadzu SPD-6AV variable-wavelength UV–visible detector and a Rikadenki multi-pen recorder (Tokyo, Japan) was used. The detector was set at 230 nm. A C_8 reversed-phase column (250×6 mm I.D.) packed with YMC-C8 (particle size 5 μm) (Yamamura Chemical Research Institute, Kyoto, Japan) was maintained at 50°C and eluted isocratically with acetonitrile–methanol–water (30:16:54, v/v/v). The pH of the solvent was maintained at 4.5 by adding acetonitrile–methanol–0.1 *M* hydrochloric acid (30:16:54, v/v/v), and the flow-rate was 1.2 ml/min. The solvent was filtered through a Nucleopore filter (pore size 0.2 μm) (Nomura Micro Science, Osaka, Japan) and degassed with a Sonifer B-12 (Branson Sonic, CT, U.S.A.) before use.

RESULTS AND DISCUSSION

Derivatization conditions

Quantitative isolation of SCFA from biological materials is difficult, owing to their volatility. Therefore, we examined the direct conversion of serum SCFA into their non-volatile 2-nitrophenylhydrazine derivatives without complicated isolation steps. Deproteinization is one of the major problems in such a direct derivatization procedure, and it can be achieved by membrane ultrafiltration or by precipitation with an organic solvent such as ethanol [9,10]. However, SCFA are usually isolated from serum or other protein-containing fluids without prior deproteinization by steam distillation, vacuum distillation or solvent extraction [10]. Remesy and Demigne [23] demonstrated that protein-bound acids, such as butyric and valeric acids in sheep plasma, were significantly lost during ultra-

TABLE I

EXTRACTION EFFICIENCIES OF THREE ORGANIC SOLVENTS FOR THE SCFA HYDRAZIDES

SCFA*	Extraction efficiency (means S.D., $n=6$) (%)		
	Ethyl acetate	Diethyl ether	Benzene
Lactic	97.3±1.4	49.2±0.6	9.9±1.5
Acetic	98.4±0.5	59.8±0.4	38.9±1.9
Propionic	102.8±0.3	87.2±0.6	74.5±1.5
Crotonic	100.8±1.2	93.5±1.2	88.6±0.8
iso-Butyric	103.1±1.0	98.9±0.7	95.7±0.7
n-Butyric	102.5±1.1	99.0±1.0	98.8±0.7
Tiglic	98.9±0.8	99.9±1.9	102.7±0.5
DL-2-Methylbutyric	100.6±0.4	100.9±0.3	101.8±0.4
3-Methylcrotonic	99.9±0.3	101.2±0.6	100.2±0.6
iso-Valeric	102.3±0.3	101.0±0.4	99.3±0.3
n-Valeric	99.4±0.4	100.6±0.6	100.5±0.5
iso-Caproic	100.3±0.3	100.4±0.4	101.8±0.6
n-Caproic	98.7±0.5	99.3±0.4	99.0±0.6

*An equimolar (50 nmol) mixture of SCFA was added to 100 μl of water and treated as described in the experimental procedure.

filtration. In our method, deproteinization was easily accomplished by the ethanol in the reaction mixture, and the protein-bound acids could be converted into their hydrazides, because the amount of ethanol (ca. 71.4%, v/v) was sufficient to denature and precipitate the protein.

Another problem is that the pH of the reaction mixture was slightly increased by basic substances in serum, resulting a decrease in the yields of the SCFA hydrazides. Therefore, 2-NPH·HCl was dissolved in 100 m*M* hydrochloric acid–ethanol (1:1, v/v) instead of water to obtain maximum yields of the hydrazides. The SCFA profile was unaffected by the amount of hydrochloric acid.

Extraction conditions

Normal serum levels of the SCFA of interest were not infrequently very low, and other acidic components were present in serum. Therefore, we investigated purification enrichment for the SCFA hydrazides using an organic solvent, where extraction depended on the partition coefficients. The long-chain fatty acid hydrazides were extracted into *n*-hexane from the reaction mixture [22], whereas the SCFA hydrazides studied were scarcely extracted with *n*-hexane. The extraction efficiencies of ethyl acetate, diethyl ether and benzene for the SCFA hydrazides are listed in Table I. Ethyl acetate seemed to be the most efficient solvent for the extraction of the SCFA hydrazides, together with some interfering substances from the reaction mixture. The extraction efficiencies of both diethyl ether and benzene were almost the same as that of ethyl acetate with the exception of lactic and acetic acid hydrazides, which occured in serum in larger amounts

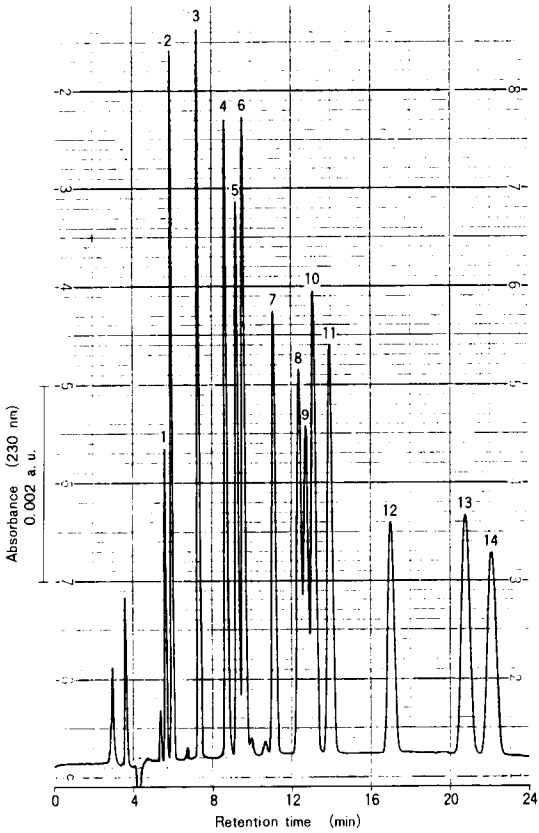

Fig. 1. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of fourteen SCFA obtained with UV detection. Peaks: 1 = lactic acid; 2 = acetic acid; 3 = propionic acid; 4 = crotonic acid; 5 = iso-butyric acid; 6 = *n*-butyric acid; 7 = tiglic acid; 8 = DL-2-methylbutyric acid; 9 = 3-methylcrotonic acid; 10 = iso-valeric acid; 11 = *n*-valeric acid; 12 = 2-ethylbutyric acid (internal standard); 13 = iso-caproic acid; 14 = *n*-caproic acid. The derivatized standard mixture of SCFA was treated according to the assay procedure. Each peak corresponds to 100 pmol.

than the other acid hydrazides. In this study, diethyl ether was chosen to minimize the interfering peaks.

To remove the long-chain fatty acid hydrazides, the neutralized reaction mixture (pH ca. 6.8) was extracted with *n*-hexane [22] prior to extraction with diethyl ether. Acidic acid hydrazides such as di- and trivalent carboxylic acid hydrazides [20] were not extracted owing to the low pK_a values of the residual carboxylic functions.

Chromatographic conditions

HPLC separation of the 2-nitrophenylhydrazine derivatives of SCFA, with the exception of unsaturated SCFA, were performed on a YMC-C8 column with methanol–water as the eluent [19]. This elution system, however, failed to provide simultaneous separation of saturated and unsaturated SCFA hydrazides. The two principal parameters for the elution volumes of fatty acid hydrazides were

TABLE II

ANALYTICAL RECOVERY AND REPRODUCIBILITY OF SCFA ADDED TO SERUM

SCFA*	Added: 1 nmol		Added: 5 nmol	
	Recovery** (%)	C.V. (%)	Recovery**	C.V. (%)
Lactic	97.8±2.4	2.5	96.8±3.7	3.8
Acetic	98.5±3.0	3.0	97.9±2.2	2.2
Propionic	99.1±2.1	2.1	102.4±3.8	3.8
Crotonic	102.1±2.8	2.7	101.8±3.0	2.9
iso-Butyric	99.5±3.4	3.4	99.8±0.9	0.9
n-Butyric	103.1±2.7	2.7	102.3±1.8	1.8
Tiglic	97.4±2.5	2.6	98.1±2.1	2.1
DL-2-Methylbutyric	101.3±1.3	1.3	99.2±1.1	1.1
3-Methylcrotonic	102.5±1.8	1.8	100.5±1.7	1.7
iso-Valeric	99.8±2.5	2.5	100.7±0.9	0.9
n-Valeric	98.7±3.1	3.1	102.1±1.5	1.5
iso-Caproic	100.5±1.2	1.2	99.6±2.8	2.8
n-Caproic	98.6±2.9	2.9	99.4±2.4	2.4

*Equimolar (1 and 5 nmol) mixtures of SCFA were added to 100 μl of human serum and treated as described in the experimental procedure.

**Recoveries are corrected for the initial presence of SCFA in serum. Data are expressed as the mean ±S.D. ($n=9$).

the number of carbon atoms and the number of unsaturated bonds in the fatty acid chain [21]. Acetonitrile and methanol have different effects on the two parameters. In acetonitrile–water, the number of unsaturated bonds seems to be of great importance in determining the elution volume, whereas in methanol–water the elution volume was relatively more influenced by the chain length. Also, derivatives of iso-isomers were eluted faster than those of the *n*-isomers [19]. Thus, by mixing acetonitrile, methanol and water in various proportions, it was possible to find the optimal overall resolution of the hydrazides of SCFA, including unsaturated SCFA.

The column temperature affected the separation of above acid hydrazides. Satisfactory resolution and favourable retention times of derivatives of thirteen SCFA and 2-ethylbutyric acid were obtained by isocratic elution with acetonitrile–methanol–water (30:16:54, v/v/v) at 50°C, as shown in Fig. 1.

Detection limits

The molar absorptivities of unsaturated SCFA hydrazides in basic medium were almost the same as those of other SCFA hydrazides [19]. The UV detection limits of HPLC, based on a signal-to-noise ratio of 2, were 200–400 fmol per injection for the SCFA, at increased detector sensitivity.

Quantitative analysis

Calibration curves were constructed by derivatizing increasing amounts of SCFA in the presence of 2-ethylbutyric acid as internal standard and by analysing

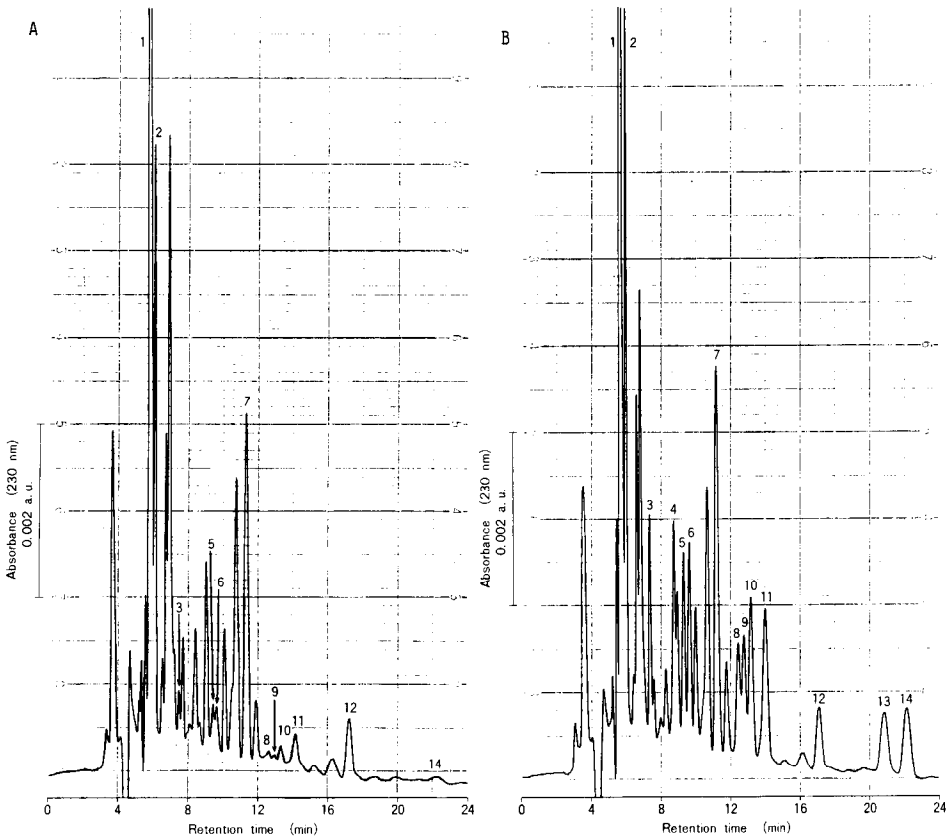

Fig. 2. Chromatograms of the derivatized SCFA in serum (A), and in serum supplemented with the SCFA (1 nmol in each) (B). For peak identification, see Fig. 1.

according to the assay procedure. The calibration test was replicated three times. From the chromatograms obtained, the relationships between the ratio of the peak heights of the acid hydrazides to that of the internal standard and the concentrations of the acids were calculated by the least-squares method. All calibration curves were linear over a wide concentration range (400 fmol to 5 nmol per injection), and the correlation coefficients of the calibration curves were in the range 0.999–1.000.

Recovery and precision

Known amounts (1 and 5 nmol) of mixtures of the SCFA were added to pooled human serum to examine the precision of SCFA measurements across a broad range of SCFA concentrations and to test the efficiency of SCFA recovery in the assay procedure. Each aliquot was analysed by nine separate measurements for

the SCFA contents. The data, summarized in Table II, indicate that the present method has satisfactory recovery and precision for practical use.

Applicability

The application of the present method to serum and serum supplemented with equimolar (1 nmol) amounts of the SCFA is illustrated in Fig. 2. Under our chromatographic conditions, the endogeneous peaks that usually occur in large amounts in sera were well resolved from the peaks of the SCFA hydrazides studied. Some of the SCFA are normally present as very small peaks or traces in the amounts of sample injected, while all the SCFA in the supplemented sample are present as easily measurable peaks. Thus, the sensitivity of the present method is sufficient for the analyses of sera of patients suffering from some metabolic disorders where the amounts of the SCFA are at least ten-fold higher than the normal values.

CONCLUSION

This study provides the first HPLC method for the simultaneous analysis of saturated and unsaturated, straight and branched, SCFA in serum. Without complicated isolation steps, the use of non-volatile hydrazine derivatives with HPLC to analyse serum samples containing SCFA has an unquestionable advantage over the GC analysis of such samples. Furthermore, it allows a good quantitative accuracy with reduced sample volume, and overall analysis time, cost and labour. Thus, the present method may be used for screening and routine monitoring of patients with several metabolic disorders.

REFERENCES

1 F.A. Hommers, J.R.G. Kuipers, J.D. Elema, J.F. Jansen and J.H.. Jonxis, Pediatr. Res., 2 (1968) 519.
2 U.G. Oberholzer, B. Leven, E.A. Burgess and W.F. Young, Arch. Dis. Child., 42 (1967) 492.
3 O. Stokke, L. Eldjarn, K.R. Norum, J. Stein-Johnson and S. Halvorsen, Scand. J. Clin. Lab. Invest., 20 (1967) 313.
4 K. Tanaka, M.A. Budd, M.L. Efron and K.J. Isselbacher, Proc. Natl. Acad. Sci. U.S.A, 56 (1966) 236.
5 J. Dancis and M. Levitz, in J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Diseases, McGraw-Hill, New York, 3rd ed., 1972, p. 397.
6 L. Przyrembel, V. Wendel, K. Becker, H.G. Bremer, L. Bruinvis, D. Ketting and S.K. Wadman, Clin. Chim. Acta, 66 (1976) 227.
7 L. Sweetman, W.L. Nyhan, D.A. Trauner, A. Merritt and M. Singh, J. Pediatr., 96 (1980) 1020.
8 L. Eldjarn, E. Jellum, O. Stokke, H. Pande and P.E. Waaler, Lancet, ii (1970) 521.
9 E. Jellum, J. Chromatogr., 143 (1977) 427.
10 T. Niwa, J. Chromatogr., 379 (1986) 313.
11 H.D. Durst, M. Milano, E.J. Kikta, Jr., S.A. Connelly and E. Grushka, Anal. Chem., 47 (1975) 1797.
12 S.T. Ingalls, P.E. Minkler, C.L. Hoppel and J.E. Nordlander, J. Chromatogr., 299 (1984) 365.
13 E. Vioque, M.P. Maza and F. Millán, J. Chromatogr., 331 (1985) 187.
14 S. Lam and E. Grushka, J. Chromatogr., 158 (1978) 207.
15 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, J. Chromatogr., 234 (1982) 121.

16 R. Farinotti, P. Siard, J. Bourson, S. Kirkiacharian, B. Valeur and G. Mahuzier, J. Chromatogr., 269 (1983) 81.
17 N. Nimura and T. Kinoshita, Anal. Lett., 13 (1980) 191.
18 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 328 (1985) 121.
19 H. Miwa, C. Hiyama and M. Yamamoto, J. Chromatogr., 321 (1985) 165.
20 H. Miwa, J. Chromatogr., 333 (1985) 215.
21 H. Miwa and M. Yamamoto, J. Chromatogr., 351 (1986) 275.
22 H. Miwa, M. Yamamoto and T. Nishida, Clin. Chim. Acta, 155 (1986) 95.
23 C. Remesy and C. Demigne, Biochem. J., 141 (1974) 85.