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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SCREENING DISORDERS OF AROMATIC ACID METABOLISM USING A MULTI-DETECTION SYSTEM

HIDEMI TODORIKI*, TOKISHI HAYASHI and HIROSHI NARUSE

National Center for Nervous, Mental and Muscular Disorders, 2620, Ogawa-Higashi-Machi, Kodaira, Tokyo (Japan)

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SUMMARY

This paper describes the use of a high-performance liquid chromatograph equipped with an ultraviolet multi-detection system for the analysis of aromatic acids to help establish a high-risk screening system for disorders of organic acid metabolism. The peak height ratios of about seventy metabolically important aromtic acids have been compiled using the multi-detection system. It may be possible to identify aromatic acids by comparing retention time and peak height ratios. The method was very effective for the diagonisis of disorders of aromatic acid metabolism.

INTRODUCTION

Since the identification of isovaleric acidaemia in 1966 by Tanaka et al. [1] using gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS), knowledge concerning organic acids in physiological fluids has been greatly extended and the number of types of organic acid disorders is still increasing.

In general, many organic acid disorders show closely similar clinical presentations, such as acidosis, ketosis, vomiting and coma, and their differential diagnosis cannot be made on clinical grounds alone. Most of the diseases reported are characterized by acute life-threatening illness in newborn babies and infants. Moreover, most surviving cases are often physically or mentally handicapped. With early diagnosis and proper therapy, patients have a better chance to survive and achieve normal physical and mental development. It is extremely important, therefore, that studies be carried out

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with a view to establishing methods for the early diagnosis and therapy of organic acid disorders.

Recently, Watts [2] reported that a study of the screening system for organic acid disorders was needed. He reported also that the disease incidence of patients with such organic acid disorders could be about 1 in 10,000, which is roughly the same as the incidence of phenylketonuria in Caucasian populations. Therefore, it will be necessary to employ a high-risk screening system for organic acid disorders, which means screening newborn babies or infants who show some clinical signs of the diseases.

Several analytical methods for organic acids in biological fluids have been reported, including paper chromatography [3], thin-layer chromatography [4], high-performance liquid chromatography (HPLC) [5—9], GC [10] and GC—MS [11—18]. Of these GC—MS has been used in the clinical diagnosis of organic acid disorders. However, the technique is expensive and not convenient for the routine analysis of a large number of clinical samples. Screening for organic acid disorders using GC—MS is only possible in a very limited number of laboratories. If a detection system able to provide good-quality information could be developed, it would be possible to screen large numbers of samples by means of the GC and HPLC methods.

This paper describes an HPLC method using a multi-detection system as a tool in screening for aromatic acid disorders.

EXPERIMENTAL

Apparatus

A Tri Rotar III high-performance liquid chromatograph equipped with three Uvidec 100-II UV spectrometers (Japan Spectroscopic, Tokyo, Japan) was used. Sample injections were performed automatically with a KSST-60 auto sample injector (Kyowa Seimitsu, Tokyo, Japan) connected to the chromatograph. HPLC separation was carried out with a stainless-steel column (250 × 4 mm I.D.) packed with LiChrosorb RP-8 (particle size 5 μm; E. Merck, Darmstadt, F.R.G.) by a balanced density slurry packing method. The operating conditions for HPLC are given in the legend to Fig. 1.

Reagents

All aromatic acids used were purchased commercially from Aldrich (Milwaukee, WI, U.S.A.), Sigma (St. Louis, MO, U.S.A.), Nakarai (Kyoto, Japan) or Wako (Osaka, Japan) and were used without purification. The other reagents and solvents were reagent grade.

Procedure

To a urine sample corresponding to 0.2 mg of creatinine, 200 μl of a 0.1 *M* solution of disodium hydrogen phosphate, containing 100 nmol of 2-hydroxy-3-naphthoic acid as internal standard, were added. The solution was diluted to 2 ml with redistilled water and then washed with 5 ml of ethyl acetate by shaking for 5 min. Urinary aromatic acids were then extracted with 5 ml of ethyl acetate by shaking for 5 min after the addition of 0.6 g of sodium

chloride and 0.5 ml of 2 *M* hydrochloric acid. The ethyl acetate layer was dried over sodium sulphate and evaporated to dryness. The residue was redissolved with two drops of N,N-dimethylformamide and diluted with six drops of 0.05 *M* phosphate buffer (pH 2.50). A 30-μl aliquot of the resulting solution was injected into the HPLC system.

Determination of creatinine

The amount of creatinine in the urine was determined by the Jaffe reaction [19]. Absorbance was measured at 520 nm with a Model UV-150-02 spectrophotometer (Shimadzu Seisakusho, Kyoto, Japan).

RESULTS AND DISCUSSION

In recent years, HPLC has been one of the fastest growing analytical techniques in the world and is used in such areas as analytical, biological and clinical chemistry, etc. This growth is due to the reliability and versatility of the separation. In general, the most popular detectors for HPLC are the UV spectrophotometer, fluorescence spectrophotometer or electrochemical detector. These detectors are highly sensitive for many compounds, but they provide little information about peak components except the retention time. Therefore, it is generally difficult to identify peak components using an HPLC system equipped with one of these detectors. This is a significant disadvantage of an HPLC system, but in order to obtain a more definitive identification of compounds, it will be necessary to develop a new type of HPLC detector, which can provide some qualitative information concerning peak components. In this investigation, a multi-detection system made up of plural detectors (in this study, three single UV detectors) was used to obtain some qualitative information. This system could facilitate the identification of aromatic acids. The three UV detectors were set at 260, 280 and 320 nm, respectively. These wavelengths lie in the UV absorption region of aromatic acids. We examined the retention times and peak height ratios of about seventy authentic samples of aromatic acids using this multi-detection system.

Most of the aromatic acids listed in Table I are normal metabolic intermediates and/or various unusual metabolites, which are known to accumulate in the urine of patients with organic acid disorders. Some groups of aromatic acids gave close retention times. These data suggest that it is not easy to identify peak components from the retention time alone. Table I indicates that even the organic acids that yielded very similar retention times can be distinguished from each other by comparing the two sets of peak height ratios. These peak height ratios must show constant values for each aromatic acid independent of its concentration, since the values represent the ratios of molecular absorption coefficients at 260, 280 and 320 nm. Thus, the data suggest that identification might be achieved by comparing the retention time and the peak height ratios, if the peak component could be considered to be single or almost single.

Tanaka and Hine [10] reported that organic acid disorders are commonly characterized by the urinary excretion of extremely large amounts of certain metabolic intermediate organic acids which are not excreted, or which are

TABLE I

RELATIVE RETENTION TIMES AND THE PEAK HEIGHT RATIOS OF AROMATIC ACIDS USING THE UV MULTI-DETECTION SYSTEM

The relative retention time, t_R (rel), was calculated for each aromatic acid relative to 2-hydroxy-3-naphthoic acid (internal standard). The values PH_{260}, PH_{280} and PH_{320} are peak heights at 260, 280 and 320 nm at 0.16, 0.16 and 0.04 a.u.f.s., respectively.

Acids	t_R (rel)	PH_{260}/PH_{280}	PH_{320}/PH_{280}
Imidazolepyruvic	0.09	0.64	0.23
5-Methylorotic	0.10	0.60	0.13
Orotic	0.10	0.54	0.07
Picolinic	0.11	2.20	0.07
Quinolinic	0.11	0.80	0.66
Isonicotinic	0.11	1.99	0.11
Nicotinic	0.12	3.07	—
Urocanic	0.14	1.07	0.04
Citrazinic	0.14	0.40	0.01
4-Hydroxymandelic	0.17	0.68	0.01
3-Hydroxymandelic	0.23	0.43	0.02
Vanillylmandelic	0.24	0.39	0.03
Homogentisic	0.25	0.15	0.13
Benzoylformic	0.33	4.15	0.15
Protocatechuic	0.37	1.90	0.30
2-Furoic	0.40	8.05	0.05
3,4-Dihydroxyphenylacetic	0.41	0.27	0.02
Mandelic	0.45	13.5	3.20
4-Hydroxyphenyllactic	0.46	0.43	0.03
Xanthurenic	0.48	2.72	3.61
4-Hydroxybenzoic	0.50	2.15	0.03
2,3-Dihydroxybenzoic	0.52	2.55	17.0
Kynurenic	0.53	3.33	13.9
Phenylpyruvic	0.53	1.20	—
4-Hydroxyphenylacetic	0.53	0.46	0.03
5-Hydroxyindoleacetic	0.53	0.61	0.44
2-Methoxymandelic	0.54	0.47	0.03
Vanillyllactic	0.54	0.30	0.03
Phthalic	0.54	0.88	0.05
Quinalidinic	0.54	1.31	18.2
3,4-Dihydroxyhydrocinnamic	0.54	0.23	0.03
Hippuric	0.54	2.89	0.18
3-Hydroxybenzoic	0.57	0.54	1.32
3-Hydroxyphenylacetic	0.57	0.46	0.02
3-Methoxymandelic	0.58	0.45	0.04
Anthranilic	0.59	3.30	19.0
Homovanillic	0.60	0.29	0.03
Caffeic	0.60	0.51	6.53
2-Hydroxyphenylacetic	0.60	0.56	0.02
Vanillic	0.61	1.71	0.14
Terephthalic	0.62	3.20	0.09
Tropic	0.63	22.7	1.00
4-Hydroxyphenylpyruvic	0.64	0.28	4.60
β-Phenyllactic	0.64	10.5	—
3-Hydroxy-4-methoxybenzoic	0.64	1.94	0.31
2-Hydroxyhippuric	0.65	0.86	2.66

TABLE I (continued)

Acids	t_R (rel)	PH_{260}/PH_{280}	PH_{320}/PH_{280}
β-3-Indolelactic	0.69	0.57	0.03
4-Hydroxycinnamic	0.70	0.27	5.30
Benzylmalonic	0.72	0.60	0.04
4-Hydroxy-3-methoxyphenylpyruvic	0.72	0.43	7.00
Ferulic	0.75	0.49	7.26
2-Methoxybenzoic	0.75	0.38	1.16
Salicylic	0.76	0.26	3.83
Benzoic	0.76	0.97	0.02
Veratric	0.77	1.76	0.17
3,4-Dimethoxyphenylacetic	0.77	0.34	0.03
5-Indolecarboxylic	0.78	0.83	0.46
3-Indoleacetic	0.79	0.60	0.02
2-Hydroxycinnamic	0.79	0.56	2.16
5-Methoxyindole-3-acetic	0.79	0.61	0.29
4-Methoxyphenylacetic	0.82	0.49	0.03
3-Methoxyphenylacetic	0.83	0.55	0.05
2-Methoxyphenylacetic	0.84	0.84	0.04
4-Methoxybenzoic	0.85	3.25	0.03
3,4-Dimethoxycinnamic	0.90	0.42	6.70
3-Indolepyruvic	0.91	0.66	11.2
3,4,5-Trimethoxycinnamic	0.93	0.37	6.10
Cinnamic	0.96	0.62	0.09
3-Indolebutyric	1.01	0.55	0.03
3-Phenyl-*n*-butyric	1.03	12.5	0.20
4-Phenyl-*n*-butyric	1.04	10.7	0.06
5-Methylindole-2-carboxylic	1.04	1.92	0.16

excreted only in small amounts into normal urine. Table II shows the lowest aromatic acid levels that are required for identification by HPLC with a multi-detection system. These results were obtained adding three different amounts of authentic aromatic acids to normal urine samples. In this investigation, a normal urine sample which gave relatively intense peaks on the chromatograms was chosen from the fifty normal urine samples investigated. The results suggest that if certain organic acids above 100 μg excreted into a urine corresponding to 0.2 mg of creatinine, almost all of the aromatic acids in Table II could be identified simply by comparing the retention times and the peak height ratios with those of authentic samples in Table I.

The level necessary for peak identification using the multi-detection system is generally much lower than that found in the urine of many aromatic acid disorders. For example, LaDu [20] reported that 1—4 mg of homogentisic acid were excreted into urine corresponding to 0.2 mg of creatinine in cases of alcaptonuria. In another type of organic acid disorder, tyrosinuria, *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetic acid are increased from 88 to 170 times normal levels [21]. Fig. 1 (left) shows a chromatogram obtained from a urine sample from a 1.5-year-old male with high blood tyrosine (38 mg/dl). The peaks marked A, A′, A″, B and C were extremely large compared with the chromatogram of a normal subject (Fig. 1,

TABLE II

ORGANIC ACID LEVELS AT WHICH IT IS POSSIBLE TO IDENTIFY ORGANIC ACIDS IN URINE

+ = identified, — = not identified.

Acids	Amount added (mg/sample)		
	0.01	0.1	1.0
4-Hydroxymandelic	—	+	+
3-Hydroxymandelic	—	+	+
Vanillylmandelic	+	+	+
Homogentisic	+	+	+
Protocatechuic	+	+	+
3,4-Dihydroxyphenylacetic	—	+	+
4-Hydroxyphenyllactic	—	+	+
Xanthurenic	+	+	+
Kynurenic	+	+	+
4-Hydroxyphenylacetic	—	+	+
5-Hydroxyindoleacetic	+	+	+
Hippuric	—	+	+
3-Hydroxybenzoic	—	+	+
Homovanillic	+	+	+
Caffeic	—	+	+
2-Hydroxyphenylacetic	—	—	+
3-Hydroxy-4-methoxybenzoic	+	+	+
2-Hydroxyhippuric	+	+	+
4-Cumaric	+	+	+
β-3-Indoleacetic	—	+	+
Ferulic	—	+	+
2-Methoxybenzoic	+	+	+
Salicylic	+	+	+
Veratric	—	+	+
5-Indolecarboxylic	+	+	+
2-Cumaric	+	+	+
4-Methoxyphenylacetic	—	+	+
3-Methoxyphenylacetic	—	+	+
Cinnamic	+	+	+

right). The retention time and the peak height ratios of each large peak seem to be close to those of *p*-hydroxyphenylpyruvic acid (A), its degradation products (A′, A″), *p*-hydroxyphenyllactic acid (B) and *p*-hydroxyphenylacetic acid (C) (Table III). It was therefore concluded that the peak components in question were the above-mentioned aromatic acids. Thus, this case was suspected as tyrosinaemia due to a deficiency of *p*-hydroxyphenylpyruvate oxidase on the basis of these results.

A few methods have been reported for the screening of organic acids by means of GC or HPLC. Tanaka and Hine [10] described a GC method of urinary organic acid analysis, which was designed to be used in screening programmes for organic aciduria; they reported that the retention indices, in terms of methylene units (MU), on two kinds of column for 163 metabolites were useful to make a diagnosis of the well defined organic aciduria. We have

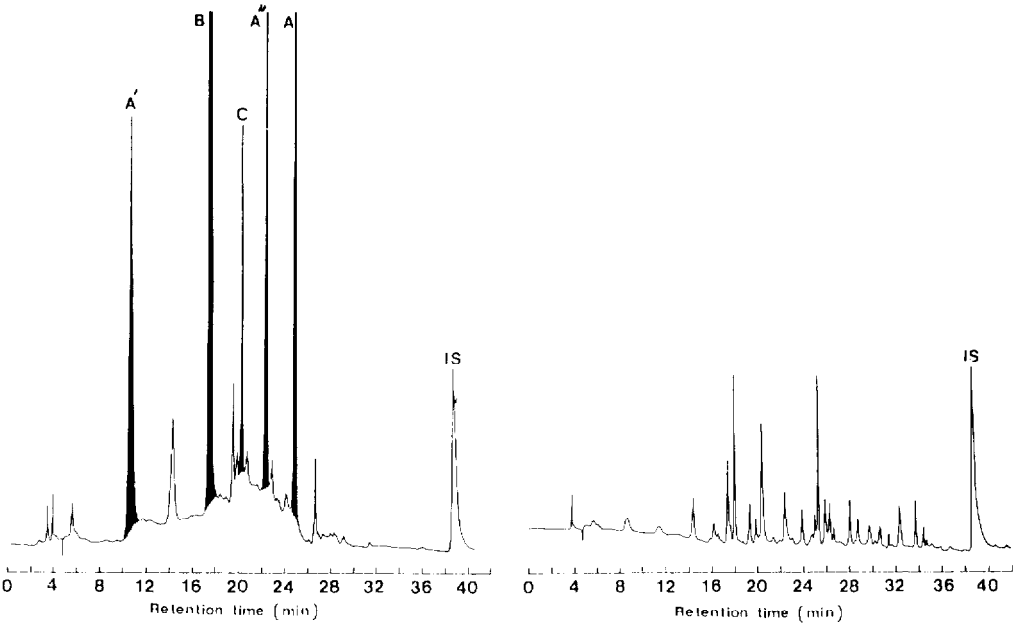

Fig. 1. High-performance liquid chromatograms of aromatic acids in urine from a patient with tyrosinaemia (left) and from a normal adult (right). The relative retention times and the peak height ratios of aromatic acids in urine on the chromatogram are shown in Table III. Operating conditions: column, LiChrosorb RP-8 (5 μm, 250 × 4 mm I.D.); column temperature, 50°C; flow-rate, 1.0 ml/min; solvent A, 0.05 *M* phosphate buffer (pH 2.5); solvent B, 80% acetonitrile; gradient, 0% B to 65% B in 40 min; detection, UV at 280 nm. Each separation was started after reconditioning the column with 150 ml of solvent A. IS = internal standard. For other peaks idenfications see Table III.

TABLE III

RELATIVE RETENTION TIME AND RESPONSE OF URINARY AROMATIC ACIDS FROM A PATIENT WITH TYROSINAEMIA USING THE UV MULTI-DETECTION SYSTEM

The values in parentheses are peak height ratios and retention times of authentic aromatic acids in Table I.

Peak	t_R (rel)	PH_{260}/PH_{280}	PH_{320}/PH_{280}	Acids
A	0.66	0.21	4.76	4-Hydroxyphenylpyruvic acid
	(0.64)	(0.28)	(4.60)	
A′	0.28	0.75	0.37	Degradation products of 4-hydroxyphenylpyruvic acid
	(0.27)	(0.70)	(0.38)	
A″	0.57	0.51	0.35	
	(0.58)	(0.51)	(0.33)	
B	0.46	0.45	0.03	4-Hydroxyphenyllactic acid
	(0.46)	(0.43)	(0.03)	
C	0.53	0.46	0.03	4-Hydroxyphenylacetic acid
	(0.53)	(0.46)	(0.03)	

tried the GC method which Tanaka and Hine [10] reported, but their method did not always give satisfactory results for the identification of some kinds of urinary organic acids. The reason why satisfactory results could not be

obtained is that the differences between MU indices obtained with the two types of GC columns were too small to identify many organic acids.

Buchanan and Thoene [7] reported the development of urinary organic acid profiling analysis of urinary organic acids utilizing two columns in series. The R_F values, together with UV absorbance ratios, were used for compound identification. Buchanan and Thoene [7] also described that the dual-column system affords better resolution of urinary organic acids than does either column separately. However, the separation of aromatic acid was not so satisfactory in comparison with results obtained by our method. In order to establish a screening system for organic acid disorders, the identification of more than one hundred organic acids would be required. However, it would be difficult to screen organic acid disorders using the dual-column method because the numbers of the different kinds of organic acids reported were not enough for identification purposes. Also, Buchanan and Thoene [7] presented 200-nm and 230-nm chromatograms obtained from a urine sample from an infant with methylmalonic aciduria. The peak on the 200-nm chromatogram, which is assigned to methylmalonic acid, is not particularly strong and the peak on the 230-nm chromatogram is absent. Therefore, it would seem difficult to obtain the peak height ratios of methylmalonic acid and to diagnose the aliphatic acid disorders using peak height ratios. Moreover, as the aliphatic acids have small molecular absorption coefficients in the UV range compared with those of aromatic acids, the peaks of aliphatic acids must be hidden by those of aromatic acids.

For the purpose of the analysis of the aromatic acid group alone, the present UV multi-detection system might be in most cases capable of spectrophotometrically resolving aromatic acids from aliphatic acids. Moreover, by means of gradient elution techniques, a good separation of the peaks on the chromatogram was achieved. The method in this investigation offers several advantages over the currently available procedure for the identification of urinary aromatic acids and was very effective for the diagnosis of aromatic acid disorders.

CONCLUSION

Organic acid disorders are characterized by acute life-threatening illness in newborn babies and infants. If these disorders are not detected early, they frequently lead to early death, or physical and mental handicaps. However, the limited availability of the GC—MS system has hindered early diagnosis of organic disorders in many areas. For the purpose of developing a new tool for the screening for aromatic acid disorders, a new HPLC detection system, the multi-detection system, was developed, and the HPLC system described above could easily identify the peak components of aromatic acids which were excreted in large amounts into the urine.

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REFERENCES

1 K. Tanaka, M.A. Budd, L. Erfron and K.J. Isselbacher, Proc. Nat. Acad. Sci. U.S., 56 (1966) 236.
2 R.W.E. Watts, in H. Naruse and M. Irie (Editors). Proceedings of the Second International Conference on Neonatal Thyroid Screening and the International Symposium on Neonatal Screening for Inborn Errors of Metabolism, Tokyo, August 16—21, 1982, Excerpta Medica, Amsterdam, 1983, p. 392.
3 J. Nordman and R. Nordman, Advan. Clin. Chem., 4 (1961) 52.
4 J.W.T. Seakins, in J. Stern and C. Toothill (Editors), Organic Acidurias, Churchill Livingstone, Edinburgh and London, 1972, p. 175.
5 I. Molnár and C. Horváth, J. Chromatogr., 143 (1977) 391.
6 D.N. Buchanan and J.G. Thoene, J. Liquid Chromatogr., 4 (1981) 1587.
7 D.N. Buchanan and J.G. Thoene, Anal. Biochem., 124 (1982) 108.
8 A. Rehman, S.C. Gates and J.W. Webb, J. Chromatogr., 228 (1982) 103.
9 E.L. Mattiuz, J.W. Webb and S.C. Gates, J. Liquid Chromatogr., 5 (1982) 2343.
10 K. Tanaka and D.G. Hine, J. Chromatogr., 239 (1982) 301.
11 E. Jellum, Trends Anal. Chem., 1 (1981) 12.
12 S.C. Gates and C.C. Sweeley, Clin. Chem., 24 (1978) 1663.
13 E.C. Horning and H.G. Horning, Clin. Chem., 17 (1971) 802.
14 S.C. Gates, N. Dendraims and C.C. Sweeley, Clin. Chem., 24 (1978) 1674.
15 J.A. Thompson and S.P. Markey, Anal. Chem., 47 (1975) 1313.
16 K. Tanaka, D.E. Hine, T.B. Lynn and T. Lowe, Clin. Chem., 26 (1980) 1874.
17 S.C. Gates, C.C. Sweeley, W. Krivit, D. DeWitt and B.E. Blaisdell, Clin. Chem., 24 (1978) 1680.
18 A.M. Lawson, R.A. Chalmers and R.W.E. Watts, Clin. Chem., 22 (1976) 1283.
19 M.Z. Jaffe, Physiol. Chem., 10 (1886) 391.
20 D.N. LaDu, in J.B. Stunbury, J.B. Wyngarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Diseases, McGraw Hill, New York, 3rd ed., 1982, p. 268.
21 L.A. Goldsmith, in J.B. Stunbury, J.B. Wyngarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Diseases, McGraw Hill, New York, 3rd ed., 1982, p. 287.