ELSEVIER

Journal of Chromatography A, 886 (2000) 297–302

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Low-capacity cation-exchange chromatography of ultraviolet-absorbing urinary basic metabolites using a reversed-phase column coated with hexadecylsulfonate

Yukio Yokoyama*, Sohei Horikoshi, Tomoko Takahashi, Hisakuni Sato

Laboratory of Analytical Chemistry, Faculty of Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya, Yokohama 240-8501, Japan

Received 31 January 2000; received in revised form 27 April 2000; accepted 27 April 2000

Abstract

A low-capacity cation-exchange HPLC method for the determination of UV-absorbing organic cations such as amino acids, histidine dipeptides, and creatinine was developed. A commercially available reversed-phase column was dynamically coated with hexadecylsulfonate, and was successfully used for the cation-exchange separation with ethylenediammonium eluting ion at pH 2.5. The coated column was enough stable for the specific use with a completely aqueous mobile phase at low and constant pH; and the day-to-day reproducibility for retention time was 0.9–1.7% of RSD (relative standard deviation). The linear relation between concentrations and detector responses (area) by using a photodiode-array UV detection at 210 nm ranged from 0.2 to 1000 μM (sample size 50 μl) for 1-methylhistidine, 3-methylhistidine, histidine, creatinine, anserine, carnosine, and homocarnosine, and from 0.5 to 2000 μM for creatine, tyrosine, and phenylalanine, with less than 5% of RSD. The UV spectrum (190–300 nm) obtained during chromatography was very indicative for each analyte. Overall recoveries were 97–104%. The developed HPLC method in conjunction with preliminary fractionation technique could be applied to the analysis of urine of patient with metabolic disorder such as phenylketonuria.

Keywords: Stationary phases, LC; Hexadecylsulfonate; Organic cations

1. Introduction

High-performance liquid chromatographic (HPLC) analyses of amino acids are essential in most biological, biomedical, clinical, and food science laboratories. Several kinds of specific instruments, so-called amino acid analyzers, are now commercially available, and are widely used for the purpose. In general, however, such specialized equipment is very expensive, and likely suitable for routine work. On the one hand, it seems that the analysis of whole amino acids is not always necessary for some specific purposes. In such cases, less expensive and simpler chromatographic systems will be favorable.

As an alternative to the conventional cation-exchange chromatography, reversed-phase ion-pair chromatography techniques have become popular to separate underivatized amino acids [1–5], where the appropriate ion-pairing agent is added to the mobile phase. In this case a highly hydrophobic surfactant is

*Corresponding author.

E-mail address: yokyuk@ynu.ac.jp (Y. Yokoyama)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00520-3

used as the ion-pairing agent, and the reversed-phase column is dynamically coated with the modifier [6–10], providing fixed-site ion exchangers that do not require addition of ion-pairing agents to the mobile phase. Such modified reversed-phase columns, having relatively lower ion-exchange capacities in general, can be used as the alternative to commercially available low-capacity columns for ion chromatography, and several advantages can be expected over the conventional ion-exchange columns: providing greater chromatographic efficiency with low-costs, more specific selectivity for ionic species, and greater flexibility in tuning ion-exchange capacity [7]. These techniques have been applied to the ion chromatography of inorganic anions [6–9] and of inorganic cations [6,10].

From the environmental viewpoint, low-capacity cation-exchange chromatography of amino acids, permitting the use of low-concentration eluent, is acceptable for routine works. In our experience at present, however, commercially available columns for cation chromatography cannot provide good selectivity for amino acid cations. Therefore, dynamically coated columns were examined for the purpose.

This paper describes a simple and low-cost cation-exchange chromatography technique for the determination of organic cations using an ODS column dynamically coated with hexadecylsulfonate with ethylenediammonium as eluting ion. The developed separation technique in conjunction with diode-array UV detection was applied to the analysis of several urinary metabolites due to inherited metabolic disorder.

2. Experimental

2.1. Reagents and samples

Phosphoric acid of biochemical grade, hydrochloric acid (20%) of super special grade, acetonitrile of HPLC grade, 1-methy-L-histidine (1MH), 3-methy-L-histidine (3MH), L-histidine, L-carnosine (Car, β-alanyl-L-histidine), L-homocarnosine (Hca, γ-aminobutyryl-L-histidine), L-tyrosine, and L-phenylalanine were purchased from Wako (Osaka, Japan). Ethylenediamine (EDA, 99.5% purity) was purchased from Aldrich (Milwaukee, WI, USA) and L-anserine (Ans, β-alanyl-1-methyl-L-histidine) was from Sigma (St. Louis, MO, USA). Sodium 1-hexadecanesulfonate (SHS), creatine (Crn), and creatinine (Cre) were purchased from Tokyo Kasei (Tokyo, Japan), and other regents were from Wako or Tokyo Kasei. All chemicals were of analytical reagent grade, and were used without further purification. Deionized water obtained through an Organo (Tokyo, Japan) G-10 mixed-bed ion-exchange cartridge with a charcoal filter was further purified by passing through a Nihon Millipore (Tokyo, Japan) Milli-Q Labo water purification system just before use.

Urine samples from patients with phenylketonuria (PKU), tyrosinemia and Lowe syndrome were furnished by Shimoshidzu National Hospital and Sanatorium, and those from healthy newborns as control were from Yokohama City University Hospital. All urine samples were stored at −30°C until use.

2.2. Apparatus

The analytical HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10AD pump, a modified Isuzu (Tokyo, Japan) AFR-111s incubator as column oven equipped with a Rheodyne (Cotati, CA, USA) 7725i syringe-loading injector with a 100-μl sample loop, a Shimadzu SPD-M10A_{VP} photodiode-array multi-wavelength UV–VIS detector, and a Fujitsu (Tokyo, Japan) FMV-5166D8 personal computer for data processing. The analytical column was a Shiseido (Tokyo, Japan) CAPCELL PAK C_{18} UG80 (150×4.6 mm I.D., particle size 5 μm, nominal theoretical plate number 13 000) reversed-phase column that was dynamically coated with SHS.

The column modification system comprised a Tosoh (Tokyo, Japan) CCPE pump, a Tosoh CM-8000 conductivity detector, a Hitachi (Tokyo, Japan) D-2000 integrator, and an Advantec (Tokyo, Japan) LS-180 water bath.

The preparative column chromatography system comprised a Rheodyne 5012 six-position rotary valve for changing the mobile phase (0.1 *M* HCl, water, 0.1 *M* NH_3), a Lab-Quatec (Tokyo, Japan) MP-311 pump, a Jasco (Tokyo, Japan) 875-UV UV–VIS detector, and an Ohkura (Tokyo, Japan) DR-1111

analog recorder. The preparative column was a glass column (100×6.5 mm I.D., 9 mm O.D., 65 mm in bed length) packed with Tosoh TSKgel SP-Toyopearl 650M (cation exchanger, 0.15 meq/ml). The detection wavelength was 254 nm and the output attenuation was 0.32 AUFS.

2.3. Column modification

Approximately, 0.2 m*M* SHS was dissolved into H_2O–CH_3CN (82.5:17.5, v/v), and then the solution was filtered through an Advantec 0.2 μm×47 mm diameter hydrophilic PTFE membrane filter by means of a vacuum filter holder unit. The coating solution was not buffered, although the equilibration of SHS depends on the pH of the mobile phase due to an ion suppression effect.

The reversed-phase column was washed and equilibrated sufficiently by passing a mixture of H_2O–CH_3CN (85:15, v/v) without surfactant in advance of the modification. Subsequently, the coating solution was pumped through the column at a flow-rate of 1 ml/min, while the background conductivity (μS/cm) of the effluent was continuously monitored. The coating temperature was kept at 30°C by immersing the column in the thermostated water bath. The effluent during coating gave the bottom conductivity of the detector, and the completion of the equilibration led to the significant increase (ca. 10 μS/cm) in the effluent conductivity. The column was then rinsed with 5 m*M* phosphoric acid (pH 2.5). The equilibrium adsorption amount of SHS was estimated by using the total volume passed through, which was 76 μmol/column corresponding to an apparent cation-exchange capacity of the column. About 7 h were necessary for the column modification.

The coating material adsorbed on the stationary phase could be removed completely by passing H_2O–CH_3CN (80:20, v/v) through the column. For this purpose, 100% CH_3CN was inadequate, because of forming the SHS crystal inside the column.

2.4. Chromatographic conditions

The analytical column successfully used was SHS-coated CAPCELL PAK C_{18} UG80; the mobile phase was 5 m*M* EDA–15 m*M* H_3PO_4 (pH 2.5) at a flow-rate of 1.0 ml/min; the column temperature was 30°C. The injection volume was 50 or 20 μl (with a 100- or 50-μl micro-syringe). The UV wavelength for detection and quantification was 210 nm, and those for identification were 190–300 nm.

2.5. Preparative chromatography for sample pretreatment

Although the preparative chromatography of urine samples is the same as described in our previous paper [11], the procedure is briefly described here. A 100-μl aliquot of urine preliminarily filtered through a 0.2-μm filter was loaded onto the preparative H^+-formed cation-exchange column and passed through the column with water. For a short while, acidic and/or neutral species were washed away from the column. After this, the mobile phase was changed to 0.1 *M* NH_3, then cationic species such as amino acids and creatinine were eluted in 1 or 2 min. The fractionation was operated with monitoring of the UV chromatographic baseline. The collected fraction, termed 'basic fraction', was freeze-dried in order to remove ammonia undoubtedly affecting the coated column, and subsequently the residue was redissolved into 1 ml of water, which led to the urine fraction ready for injection diluted 10-fold versus intact urine.

As an alternative to the freeze-drying process, acidification of the ammonia fraction with H_3PO_4 is also acceptable.

3. Results and discussion

3.1. Analytical column selection and evaluation

In the reversed-phase separation using ODS columns, in general, the addition of at least 5 or 10% (v/v) water-soluble organic solvent, such as acetonitrile, to the mobile phase is recommended to avoid decreasing the ODS activity, i.e., column hydrophobicity. However, the addition of such organic solvent to the mobile phase is unacceptable for the intended chromatographic separation using a surfactant-coated ODS; otherwise, the surfactant will gradually be desorbed from the stationary phase. Although various kinds of ODS–silica columns are commer-

cially available from different suppliers, their physical durability under the organic-free eluent conditions and their applicability to the specific purpose seemed to be different from one brand to another.

In this study, four commercially available, conventional silica-based (a Tosoh TSKgel ODS-80Ts (150×4.6 mm I.D., 5 μm), a GL Sciences (Tokyo, Japan) Inertsil ODS-2 (150×4.6 mm I.D., 5 μm), a Nomura Chemical (Seto, Japan) Develosil ODS-UG-5 (150×4.6 mm I.D., 5 μm), and CAPCELL PAK C_{18} UG80) and two polymer-based (a Showa Denko (Tokyo, Japan) Asahipak ODP-50 (150×4.6 mm I.D., 5 μm, polyvinylalcohol base polymer) and a Tosoh Octadecyl-2PW (150×4.6 mm I.D., 5 μm, polyhydroxymethacrylate base polymer)) reversed-phase columns were examined to find the one giving acceptable durability and resolution.

Each ODS–silica column dynamically coated with SHS individually provided an excellent selectivity for amino acid cations, and gave relatively good separation for hydrophilic amino acids. Nine hydrophilic amino acids were separated in the order of Asn, Asp, Ser, Gly, Gln, Glu, Thr, Ala and Pro, with retention times ranging from 5 to 30 min by an isocratic elution with 5 m*M* H_3PO_4, which was very similar to the results obtained by isocratic ion-pair separation [5]. Such good selectivity for amino acids could not be obtained when using commercially available low-capacity cation-exchange columns for ion chromatography of inorganic cations.

Despite the above merits, however, we found that the coated ODS–silica columns were easily damaged by changing the eluent pH under such completely organic-free conditions, even within acidic pH range, which led to reducing the column hydrophobicity probably due to some twisting and/or laying-down effects of octadecyl chains. On the other hand, the polymer-based ODS columns were stable in use under various eluent pH conditions, but serious shrinking effects on the base polymer were often observed after passing the coating solution, which led to a dead volume at the column inlet, probably due to the presence of surfactant.

Among the tested columns, the SHS-coated CAPCELL PAK C_{18} column was found to give satisfactory performance for separating amino acid cations with a completely organic free eluent at low pH. The ion-exchange capacity of the column was changed by changing the CH_3CN contents in the coating solution [7]. The chemically adsorbed SHS amounts were approximately 120, 76, and 44 μmol per column with 15, 17.5, and 20% (v/v) of CH_3CN contents, respectively. Among these, the column coated with the 17.5% (v/v) CH_3CN solution gave an intended cation-exchange capacity for the separation, providing adequate retention times between 5 and 12 min for the nine hydrophilic amino acids with 5 m*M* H_3PO_4 (pH 2.5) eluent. This SHS-coated column worked well during this study for 3 months or over without serious change in performance with keeping eluent pH constant at 2.5 (with H_3PO_4).

3.2. Separation and quantification

Fig. 1 shows a chromatogram of ultraviolet absorbing amino acids, i.e., 1MH, Crn, 3MH, His, Cre, Ans, Car, Hca, Tyr, and Phe, eluted with 5 m*M* EDA divalent cation at pH 2.5 adjusted by H_3PO_4 (15 m*M*) and monitored at UV 210 nm. The separation was very good with a theoretical plate number of ca. 7500 calculated with the peak of Hca. Other basic amino acids such as Lys and Arg were also separated from these analytes, and had little influence on the quantification because of their UV transparency.

The chromatography with standard solutions was

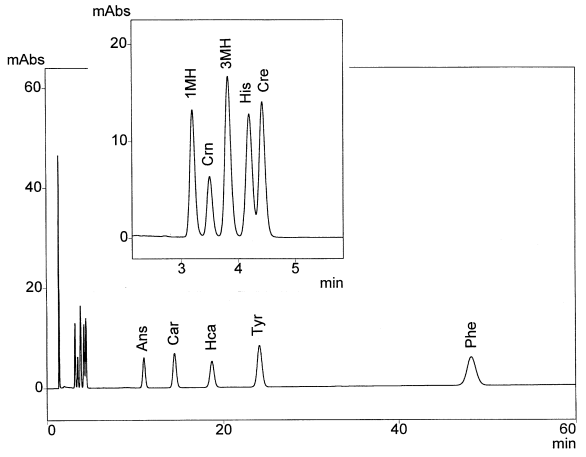

Fig. 1. Standard chromatogram of the analytes. Column, SHS-coated CAPCELL PAK C_{18} UG80; eluent, 5 m*M* EDA–15 m*M* H_3PO_4 (pH 2.5); concentration, 1MH, Crn, 3MH, His, Cre, Ans, Car, Hca=20 μ*M*; Tyr, Phe=50 μ*M*; injection volume, 20 μl; flow-rate, 1.0 ml/min; detection, UV 210 nm.

very reproducible and quantitative. Relative standard deviation (RSD) of retention time for each analyte in different concentrations ranged between 0.1 and 1.1% ($n=5$ each) for within-day repeatability and between 0.9 and 1.7% for day-to-day reproducibility. RSD of area ranged between 1.6 and 3.1% for within-day and between 2.2 and 3.6% for day-to-day for the analytes (sample size 20 μl×50 μ*M* each). Further quantification data are summarized in Table 1.

3.3. Recoveries

Chromatographic recoveries were measured using a standard mixture containing 1MH, Cre, Ans, Car, Hca, and Phe (10 m*M* each). A 100-μl aliquot of the mixture was subjected to the preparative chromatography followed by the analytical chromatography. Overall recoveries ranged between 97 and 104%.

3.4. Application to urine of patient with inherited metabolic disorder

The method was applied to the analyses of urines from patients with several inherited metabolic disorders such as PKU, tyrosinemia, and Lowe syndrome.

Fig. 2 shows a typical chromatogram of the basic fraction from a urine of patient with PKU. The peaks of Cre, clinically important metabolite, and Phe, marker of the disease, appeared at 4.64 and 47.45 min, respectively. The on-flow UV spectra between 190 and 300 nm for individual analytes under the present chromatographic condition were very helpful for the peak identification, although several peaks appeared within 6 min for each chromatogram. From the individual concentration data, the creatinine ratio [2,11,12], i.e., Phe (m*M*)/Cre (m*M*), could be easily determined to be 0.34. The ratios for the five PKU urines examined here ranged from 0.17 to 0.62, which were significantly large compared with normal levels (<0.015).

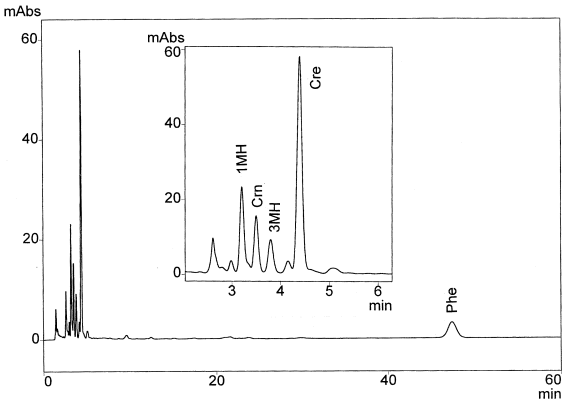

Fig. 2. Chromatogram of the basic fraction from a urine of patient with PKU (corresponding to 10-fold dilution of intact urine). Conditions were the same as in Fig. 1 with 50-μl injection.

Similarly, the Tyr/Cre ratio in tyrosinemia was significantly larger than that in control. In case of Lowe syndrome (Fig. 3), the creatinine ratios for Tyr and for Phe were both in high levels. In addition, Ans and Car in high concentrations were also detected in one of PKU urines, i.e., PKU (5), which were confirmed both by their UV spectra and by co-injection technique.

These analytical results are summarized in Table 2. The values were enough reliable with RSDs under 5% ($n=3$).

Table 1
Quantification data for the analytes

Analyte	Linear range[a] (μ*M*)	RSD (area)[b] (%)	r^2	Detection limit[c] (μ*M*)
1MH	0.2–1000	1.2–1.6	0.9998	0.02
3MH	0.2–1000	1.2–3.1	0.9998	0.02
His	0.2–1000	1.2–3.0	0.9998	0.02
Crn	0.5–2000	1.5–3.5	0.9998	0.05
Cre	0.2–1000	0.2–3.6	0.9998	0.02
Ans	0.2–1000	0.7–2.1	0.9998	0.02
Car	0.2–1000	0.9–4.7	0.9998	0.02
Hca	0.2–1000	1.1–3.6	0.9998	0.02
Tyr	0.5–2000	0.8–5.1	0.9999	0.1
Phe	0.5–2000	0.7–2.5	0.9997	0.1

[a] Sample size was 50 μl each.
[b] Deviation was dependent on the analyte concentration. The smaller concentration could give larger deviation.
[c] $S/N=3$.

4. Conclusion

The SHS-coated ODS columns were very stable; and the column performance was unchanged for fairly long period under constant acidic pH conditions, e.g., with pH 2.5 eluent. Such low-capacity

Table 2
Analytical results of urinary basic metabolites

Metabolic disease	Concentration in intact urine (m*M*)										Creatinine ratio (m*M*/m*M*)	
	1MH	3MH	Crn	His	Cre	Ans	Car	Hca	Tyr	Phe	Tyr/Cre	Phe/Cre
PKU (1)	0.57	0.31	0.88	0.10	1.65	–[a]	–	–	0.035	0.56	0.021	0.34
PKU (2)	0.31	0.38	0.91	0.075	3.38	–	0.008	–	0.024	1.08	0.007	0.32
PKU (3)	0.033	0.10	0.35	0.014	0.27	–	0.018	–	0.011	0.17	0.041	0.62
PKU (4)	0.020	0.22	0.44	0.044	1.00	0.005	0.041	0.018	0.014	0.30	0.014	0.30
PKU (5)	0.91	0.31	0.89	0.094	1.78	0.84	0.078	0.015	0.013	0.30	0.007	0.17
Tyrosinemia	0.022	0.22	0.47	0.031	0.91	0.005	0.084	–	0.10	0.016	0.12	0.018
Lowe syndrome	0.049	2.59	1.24	0.079	1.39	–	–	–	0.50	0.36	0.36	0.26
Control (1)	0.22	0.26	0.40	0.072	2.74	–	–	–	0.024	0.015	0.009	0.006
Control (2)	0.013	0.25	0.90	0.052	1.38	–	–	–	0.030	0.008	0.022	0.006
Control (3)	0.039	0.051	0.067	0.028	0.82	–	–	–	0.006	0.006	0.007	0.007

[a] –, not detected or not identified.

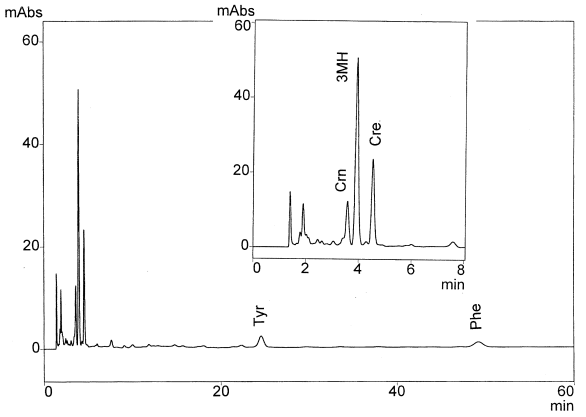

Fig. 3. Chromatogram of the basic fraction from a urine of patient with Lowe syndrome (corresponding to 100-fold dilution of intact urine). Conditions were the same as in Fig. 1 with 50-μl injection.

cation-exchange columns could provide high resolution and excellent selectivity for amino acid cations. Although there are some limitations in the chromatographic conditions acceptable, the developed HPLC method in conjunction with the preparative chromatography is considered to be practical and useful for the analysis of urinary UV-absorbing organic cations, including amino acids, histidine dipeptides, and creatinine, and to be applicable to the chemical diagnosis of inherited metabolic disorders.

Acknowledgements

This work was supported, in part, by a grant from the Yokohama Kogyo-kai Foundation (Yokohama National University). The authors wish to thank Mr. Y. Ohtsu of Shiseido Research Center (Yokohama, Japan) for helpful discussion.

References

[1] J. Saurina, S. Hernández-Cassou, J. Chromatogr. A 676 (1994) 311.
[2] Y. Yokoyama, O. Ozaki, H. Sato, J. Chromatogr. A 739 (1996) 333.
[3] M. Dunnett, R.C. Harris, J. Chromatogr. B 688 (1997) 47.
[4] N. Tateda, K. Matsuhisa, K. Hasebe, N. Kitajima, T. Miura, J. Chromatogr. B 718 (1998) 235.
[5] K.N. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, J. Chromatogr. A 833 (1999) 147.
[6] R.M. Cassidy, S. Elchuk, Anal. Chem. 54 (1982) 1558.
[7] R.M. Cassidy, S. Elchuk, J. Chromatogr. Sci. 21 (1983) 454.
[8] F.G.P. Mullins, Analyst 112 (1987) 665.
[9] K. Ito, Y. Ariyoshi, F. Tanabiki, H. Sunahara, Anal. Chem. 63 (1991) 273.
[10] W. Hu, T. Takeuchi, H. Haraguchi, Anal. Chim. Acta 267 (1992) 141.
[11] Y. Yokoyama, H. Sato, M. Tsuchiya, H. Kakinuma, J. Chromatogr. 566 (1991) 19.
[12] Y. Yokoyama, M. Tsuchiya, H. Sato, H. Kakinuma, J. Chromatogr. 583 (1992) 1.