Journal of Chromatography, 228 (1982) 273—278
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1126

Note

Rapid analysis of 3-methylhistidine in urine, plasma, muscle and amniotic fluid with a single high-performance liquid chromatographic system but with different ion-pairing reagents

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(First received July 13th, 1981; revised manuscript received October 1st, 1981)

After the initial demonstration [1] that ion-paired reversed-phase high-performance liquid chromatography (HPLC) could be successfully applied to the analysis of amino acids, peptides and proteins, this technique has been applied to the analysis of a variety of samples [2—5]. These examples, together with more detailed studies on ion-pairing reagents [6—9], demonstrated that the addition of hydrophilic (for example, phosphoric acid) or hydrophobic (for example, hexanesulphonic acid) reagents to the mobile phase allowed the chromatographer to manipulate the retention time of a peptide or protein sample on a given hydrocarbonaceous support. In a similar manner the retention of dibasic amino acids relative to other amino acids can be selectively increased by the addition of a suitable hydrophobic ion-pairing reagent to the mobile phase [10, 11]. For example, hydroxylysinonorleucine, one of the major constituents of collagen crosslinks can be analysed in a collagen hydrolysate using a μBondapak C_{18} column with a mobile phase which contains sodium dodecylsulphate [12].

The application of hydrophobicity parameters for the prediction of retention of a series of amino acids and peptides has been placed on a sound theoretical basis [13—15]. The retention of these solutes on a non-polar stationary phase is a function of the hydrophobicity of the peptide-paired ion—solvent

0378-4347/82/0000—0000/$02.75

hydration complex [15]. This effect can be demonstrated with a series of mobile phases which contain alkylsulphonates of increasing carbon-chain length as ionic modifiers. Therefore, it is possible to progressively increase the retention of a solute by simple changes in the composition of the mobile phase [6—9, 14, 15]. Other components in a biological sample will be affected to an extent which depends on the balance of polar and non-polar groups present in each solute. It is usually possible to achieve a narrow "window" for the analysis of a single component of a complex mixture by the judicious choice of ion-pairing reagent. In an effort to evaluate this approach, we have synthesised the homologous series of alkylsulphonates, $CH_3(CH_2)_nSO_3Na$, where n = 1—11, using standard procedures [16]. These ion-pairing reagents have allowed the development of a versatile chromatographic system for the analysis of amino acid and peptide samples in complex biological mixtures.

The muscular dystrophies are a group of diseases characterised by a relentless progress of increasing muscle weakness. For example, a striking feature of the disease is the extensive loss of sarcoplasmic contractile proteins and their replacement by fat and connective tissue [17]. 3-Methylhistidine is closely associated with muscle metabolism in the body as the vast majority of this amino acid is present in the myofibrillar protein of skeletal muscle [18]. Methylation of histidine occurs after its incorporation into actin and myosin [19] and after catabolism of these proteins the liberated 3-methylhistidine is not recycled but quantitatively excreted in the urine [20]. The total amount of 3-methylhistidine excreted in the urine has therefore been proposed as an index of muscle protein catabolism [20]. This report describes an HPLC method for the analysis of 3-methylhistidine in four different biological samples.

EXPERIMENTAL

Materials

All reagents used were of highest purity available (A.C.S. Certified grade). Boric acid, β-mercaptoethanol, potassium hydroxide (Fisher Scientific, Pittsburgh, PA, U.S.A.), *o*-phthalaldehyde (Eastman Kodak, Rochester, NY, U.S.A.), Brij 35, 30% solution (Pierce, Rockford, IL, U.S.A.), and ethanol (U.S. Industrial Chemicals, New York, NY, U.S.A.), were used in preparing the derivatization reagent. L-3-Methylhistidine, L-1-methylhistidine and L-histidine were purchased from Sigma (St. Louis, MO, U.S.A.). The alkylsulphonates were synthesised using the procedure of Truce and Norrell [16].

Methods

The *o*-phthalaldehyde reagent was prepared by dissolving 30 g of boric acid in 1.0 l of deionized water, adjusting the pH to 10.4 with potassium hydroxide pellets, filtering through a 0.45-μm aqueous filter (Millipore, Bedford, MA, U.S.A.) and adding 1.0 ml of 30% (w/v) aqueous solution of Brij 35, then transferring the solution into a dark glass bottle. Separately, a solution containing 600 mg of *o*-phthalaldehyde and 200 μl of β-mercaptoethanol in 10 ml of ethanol was added to the borate solution and stored under nitrogen. The reagent was prepared daily.

A stock solution of 5 m*M* sodium alkylsulfonate, pH 3.2 (pH adjusted with glacial acetic acid) in the deionized water was degassed prior to use as the mobile phase in the chromatograph.

A standard solution of 3-methylhistidine was prepared in deionized water and analyzed by HPLC. When not in use, the solution was stored at 4°C.

Apparatus

A Model 6000A solvent delivery system and U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) were used with the HPLC system. The fluorescence detector was a Model 420 using an excitation filter of 340 nm and an emission filter of 440 nm (Waters Assoc.). The HPLC column used was stainless steel (300 × 4 mm I.D.), μBondapak C_{18}, particle size 10 μm, with a guard column, 2.5 cm long and 0.5-ml volume capacity, packed with Bondapak Phenyl/Corasil, particle size 37—50 μm, and a post-column mixing chamber, all from Waters Assoc.

The emission of 440 nm was monitored at a chart speed of 5 mm/min on a 10-mV linear recorder B-5000 OmniScribe (Houston Instrument, Austin, TX, U.S.A.). The peak heights were measured.

RESULTS AND DISCUSSION

Gas—liquid chromatography (GLC) has proved to be a versatile technique for the analysis of amino acid derivatives, particularly when coupled to mass spectrometric detection [21]. However, HPLC has become an attractive alternative, particularly as highly selective separations can be achieved by the addition of different ion-pairing reagents to the mobile phase. An important advantage of many HPLC separations, unlike GLC methods, is that the underivatised amino acid can be analysed directly. Recently rapid and sensitive analytical separations of 3-methylhistidine in urine samples were reported by Friedman et al. [22], Ward et al. [23] and Wassner et al. [24]. Fig. 1 gives an example of the high efficiency separation of histidine, 1-methylhistidine and 3-methylhistidine that can be achieved with a mobile phase containing 5 m*M* sodium hexanesulphonate. In this separation the solutes were monitored by fluorescent detection after post-column derivatisation with *o*-phthalaldehyde. In our previous study on the analysis of 3-methylhistidine by this procedure [22], we showed that the analytical statistics for the assay were excellent, with close agreement between measurements at different concentrations.

For this assay to be useful as an index of muscle catabolism in disease states it is necessary to determine the relationship between urinary levels of 3-methylhistidine, the corresponding levels in plasma and values obtained for selected muscle biopsy samples. In addition, the assay of amniotic fluid samples is of importance as these measurements may allow pre-natal screening and diagnosis of congenitally transmitted muscle diseases. In an effort to develop a single chromatographic system which would allow the analysis of 3-methylhistidine in a range of biological samples, we investigated the effect of different alkylsulphonates on the retention of this solute on a μBondapak C_{18} column. Fig. 2 shows the analysis of a muscle biopsy sample with mobile

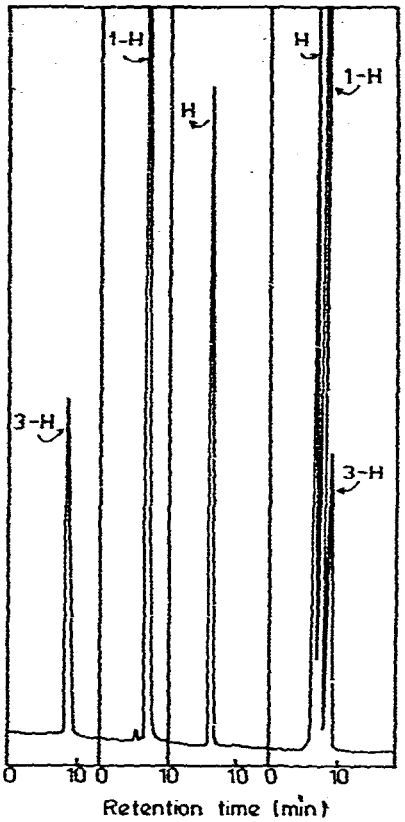

Fig. 1. Reversed-phase HPLC separation of 3-methylhistidine (3-H), 1-methylhistidine (1-H) and histidine (H) and a mixture of the three standards. The analysis used a μBondapak C_{18} column (300 × 4 mm I.D.) and 5 m*M* sodium hexanesulphonate, pH 3.2 (pH adjusted with glacial acetic acid) as the mobile phase. The flow-rate was 0.8 ml/min and other chromatographic parameters were as previously described. The samples were detected by post-column fluorescent derivatisation which used a mixing chamber with o-phthalaldehyde (1 ml/min) and a fluorescent detector (Waters Model 420), excitation 340 nm and emission 440 nm.

phases which contain C_4—C_9 sulphonates. With the increasing chain length of the alkylsulphonate the retention of 3-methylhistidine progressively increases. It is only with nonylsulphonate as the ion-pairing reagent that the solute of interest is sufficiently resolved from contaminant peaks for quantitation. The large optical density peak eluting at 38 min with octanesulphonate is retained indefinitely with the nonylsulphonate system.

As is shown in Fig. 3 the alkylsulphonate required to give resolution of 3-methylhistidine from other solutes was different for each biological sample. With a urine sample, hexanesulphonate gave a good separation of 3-methylhistidine, while plasma required heptanesulphonate, amniotic fluid octanesulphonate and muscle tissue nonanesulphonate. In each case the alkylsulphonate gave a window in which the amino acid could be eluted free of contaminating material. As was described in the earlier publication [22], 3-methylhistidine was identified by the chromatography of an authentic sample and by

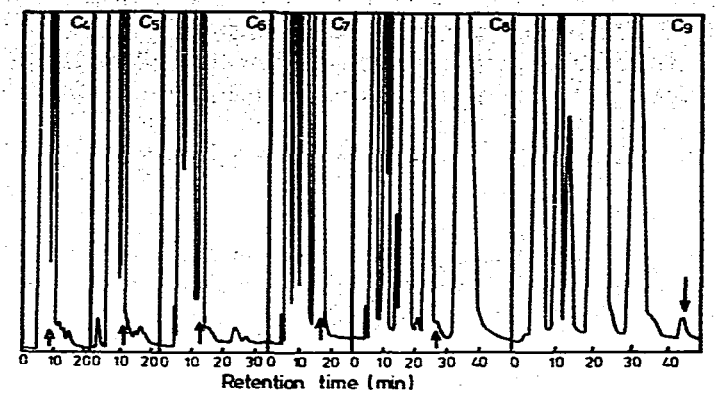

Fig. 2. Separation of a muscle sample on a μBondapak C_{18} column with mobile phases which contained different alkylsulphonates (C_xSO_3Na, where C_x is listed in the figure, 5 m*M* in each case). A 10% solution of trichloroacetic acid was added to the sample (1:9, v/v), the sample was centrifuged, the supernatant adjusted to pH 3.5 with 10% trichloroacetic acid and a 1.5-μl aliquot was injected for the HPLC analysis. The elution time expected for 3-methylhistidine is shown by the arrow.

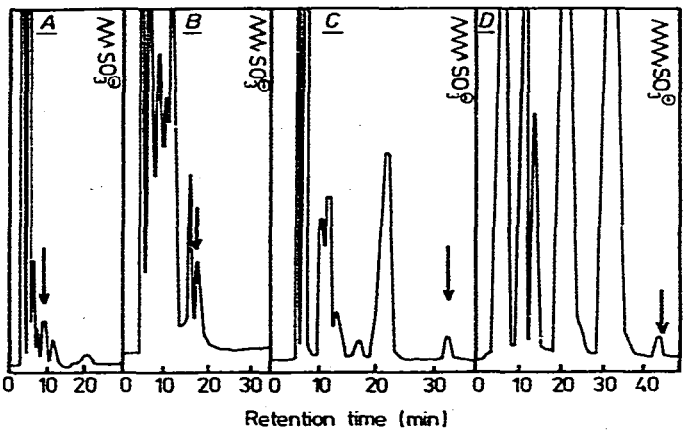

Fig. 3. Analysis of 3-methylhistidine in a 5-μl urine sample (A); 15-μl plasma sample (B); 15-μl amniotic fluid samples (C); and 2.5-μl muscle sample (D). The chromatographic conditions used in Fig. 1 were used except that the following alkylsulphonates were used: (A) hexanesulphonate; (B) heptanesulphonate; (C) octanesulphonate; (D) nonanesulphonate. In (D) 1.5% acetonitrile was added to the mobile phase, in all other cases an aqueous solution of the ion-pairing reagent was used. The preparation of the samples followed the procedure described in the legend to Fig. 2.

peak enhancement techniques. An important advantage of this procedure is that it allows the direct analysis of the biological sample, after a simple deproteinising step has been carried out.

In conclusion, this report has shown that reversed-phase HPLC with ion-pairing reagents is an extremely versatile chromatographic technique. The technique allows the analysis of urine, plasma, serum and muscle samples with a single chromatographic system, provided a suitable alkylsulphonate is

added to the mobile phase. In addition, we are currently using these chromatographic systems to establish the relationship between the levels of 3-methylhistidine in the different samples and use of these values as an index of muscle catabolism in vivo.

ACKNOWLEDGEMENTS

This study is partially supported by a grant from the Medical Research Council and National Heart Foundation of New Zealand and National Institutes of Health, USA, Grant No. NICHD-HD-13507-02.

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