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Short Communication

Separation of *o*-phthalaldehyde–mercaptoethanol derivatives of amino acids from blood plasma on reversed-phase Nova-Pak C_{18} cartridges

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ABSTRACT

A separation of 25 *o*-phthalaldehyde–mercaptoethanol derivatives of primary amino acids in plasma prepared from human blood has been developed for Waters 10 cm × 0.8 cm I.D., 4-μm Nova-Pak C_{18} Radial-Pak cartridges. A binary gradient system with solvent-switching capability for the A pump is required. Computer methodologies have been utilized to develop mobile phase mixtures of phosphate buffer (pH 6.9), water, methanol and tetrahydrofuran. Advantages of the method include simple sample preparation, fast turnover time (67 min including the pre-column Autotag derivatization procedure) and exceptional column durability (several hundred analyses).

INTRODUCTION

Numerous reversed-phase C_{18} methods for the separation of *o*-phthalaldehyde–mercaptoethanol (OPA–ME) derivatives have been published [1–4]. Earlier, we reported a separation suitable for analysis of primary amino acids from human blood plasma on Waters C_{18} Resolve non-endcapped, 5-μm, 10 cm × 0.8 cm I.D. Radial-Pak cartridges [5]. Radial compression cartridges have several advantages over steel columns: larger bed volumes, lower operating pressures, less susceptibility to operating conditions and longer life. The Autotag OPA–ME automated derivatization procedure requires an additional coil between the injector and the column, ramped interruption of flow before each injection and injection of a small aliquot of alkaline 0.4 *M* borate (8 μl at pH 10.4). Especially under these conditions, the radial compression cartridges would be expected to give better performance.

Over the course of many analyses, as we reported earlier, shifts of retention times and selectivity changes occur with the Resolve columns, which are not duc

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to solvent preparation or temperature changes. While all important known components for our particular study were resolved (including branched-chain and aromatic amino acids), incomplete separation of Gln and His, as well as incomplete separation and excessive bandwidths for Cit, Gly, Thr and Meh were disadvantages. Further, in plasma of alcoholic hepatitis patients a prominent band (ethanolamine) appeared which sometimes co-eluted with tryptophan. Here we report an improved separation using Waters endcapped, 4-μm, 10 cm × 0.8 cm I.D. Nova-Pak C_{18} cartridges.

EXPERIMENTAL

Equipment and materials

The Waters binary-gradient high-performance liquid chromatograph configured for Autotag automated precolumn derivatization was used as previously described [5]. A Waters 10 cm × 0.8 cm I.D. Nova-Pak C_{18}, 4-μm Radial-Pak analytical cartridge and a Nova-Pak guard column were used in place of the Resolve-type guard column and analytical cartridge used previously (all from Millipore-Waters, Milford, MA, USA). Chemicals were obtained from sources as previously described.

Conditions for the multi-solvent gradient separation were determined by methods discussed previously, using retention times acquired with gradient runs, in conjunction with computer programs DryLab G (LC Resources, Lafayette, CA, USA) [6], RTGRAPH and LCSIM [5,7].

Mobile phase preparation

Solvent $A_{N(3.9)}$: 0.025 *M* NaH_2PO_4, 0.025 *M* Na_2HPO_4, 3.9% (v/v) tetrahydrofuran, 1.1% (v/v) methanol and 95% (v/v) water. Solvent D_N: 0.035 *M* NaH_2PO_4, 0.035 *M* Na_2HPO_4, 5% (v/v) tetrahydrofuran and 95% (v/v) water. Solvent A_0: 0.025 *M* NaH_2PO_4, 0.025 *M* Na_2HPO_4, 4% (v/v) methanol and 96% (v/v) water. These three solvents had a pH of 6.9 and were used with the A pump.

Solvent B: 65% (v/v) methanol and 35% (v/v) water.

Analytical gradient scheme

Before each run, the column was equilibrated for 7 min ($A_{N(3.9)}$–B, 68:32) at 1.2 ml/min (the flow-rate used throughout the separation). At the beginning of each run the Autotag procedure was carried out as previously described [5,7]; the flow was initiated with $A_{N(3.9)}$–B (75:25). Solvent switches were programmed at 9.1 min (D_N–B) and at 16.9 min (A_0–B). The dead volume between the solvent switch valve and the pump inlet was approximately 0.5 ml.

Linear gradient segments were defined by the following events (time in min, followed by percentage solvent B): (14, 25); (15, 35); (19, 50); (20, 64); (22, 64); (24, 79); (26, 80); (30, 84); (34.5, 93); (41, 100); (49, 100); (52, 32). The flow-rate was decreased (1-min ramp) after a 7-min equilibration for the next run to 0 at 60 min.

Sample preparation

Blood plasma was separated from erythrocytes by centrifugation at 1500 *g* and stored at −70°C. On thawing, 0.25 ml of plasma was deproteinized by mixing with 2 ml of methanol. Precipitated proteins were removed by centrifugation (20 min at 1500 *g*), and 0.5 ml of the supernatant was diluted with 2 ml of buffer [pH 2.2; 0.05% (v/v) trifluoroacetic acid, 5% (v/v) methanol, 94.95% (v/v) water]. Samples were filtered into WISP vials with Millex filters (Millipore-Waters). Samples thus prepared could be stored at −70°C without observable changes. For standards, a solution of 25 amino acids at 0.1 m*M* was substituted for plasma, and all preparative steps were carried out, as with the other samples.

For analysis of cysteine + cystine, R–SS–R groups can be reduced with dithiothreitol then R–SH alkylated with iodoacetate [8].

Notes on the separation

A higher volume fraction of solvent B in the mobile phase improved resolution of Cit-Gly, Tau-Ala and Trp-Met, but was detrimental for resolution of other critical pairs. A higher concentration of tetrahydrofuran was detrimental to Ser-His and Trp-Met resolution. A higher concentration of phosphate was detrimental to Ser-His resolution and generally increased retention times.

RESULTS

Chromatograms from plasma and 25 standards separated by our method for Nova-Pak cartridges are shown in Fig. 1. Bandwidths are only slightly larger than

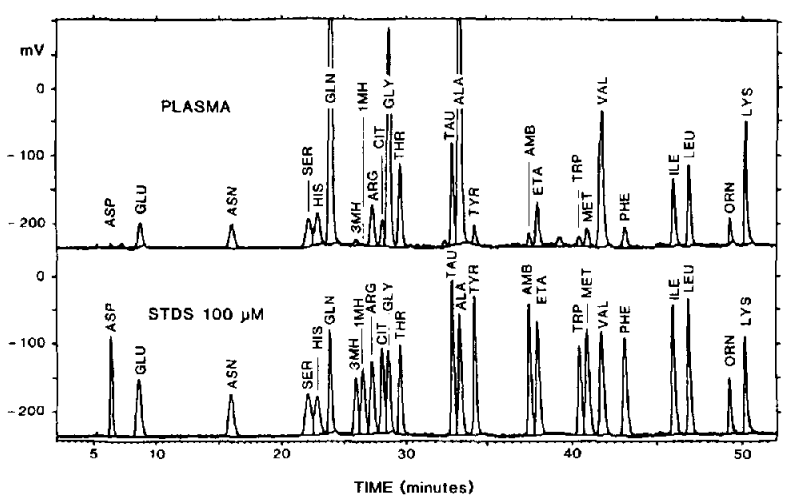

Fig. 1. Separation of OPA–ME derivatives of amino acids, commonly found in human blood plasma, on a Waters C_{18} Nova-Pak analytical radial compression cartridge. For each standard amino acid 18 pmol were injected, corresponding to a concentration of 0.1 m*M* in plasma. For abbreviations, see Table I.

desirable for Asp, Ser and His derivatives, an improvement over the previous Resolve separation. The two methylhistidine isomers are now separated. However, since methylhistidines, as well as Asp, are of very low concentration in plasma, statistical studies involving small differences in their concentrations will require that the plasma be diluted less (our injection volume was 8 μl, and 18 pmol injected corresponded to a plasma concentration of 0.1 m*M*). In the samples assayed, sample overload was not a problem.

The ethanolamine derivative is consistently resolved, eluting just after AMB. Other derivatives important for our studies of plasma primary amino acids in liver disease, including the branched-chain and aromatic amino acids, Met, Trp, etc., are separated adequately, with a complete Autotag cycle time of only 67 min.

TABLE I

RETENTION TIMES FOR OPA–ME DERIVATIVES USING A NOVA-PAK REVERSED-PHASE C_{18} CARTRIDGE

Common derivatives	Abbreviation	Retention time (min)	Other derivatives	Retention time (min)
Aspartate	Asp	7.2	Phosphoserine	6.4
Glutamate	Glu	9.8		
Alkylated cysteine	Cys	10.5	Aminoadipic acid	14.3
Asparagine	Asn	16.9		
Serine	Ser	22.4		
Histidine	His	23.1		
Glutamine	Gln	23.9	Unknown[a]	24.3
3-Methylhistidine	1MH	25.9	Phosphoethanolamine	25.8
1-Methylhistidine	3MH	26.4	S-Adenosylmethionine	26.6
Arginine	Arg	27.1		
Citrulline	Cit	27.9		
Glycine	Gly	28.4	Carnosine	29.2
Threonine	Thr	29.3	Anserine	29.3
Taurine	Tau	32.6	β-Alanine	32.2
Alanine	Ala	33.1	Hypotaurine	33.0
Tyrosine	Tyr	33.9	γ-Aminobutyric acid	33.7
α-Aminobutyrate	AMB	37.1	β-Aminobutyric acid	34.0
Ethanolamine	ETA	37.4	Cystathionine I	39.3
Tryptophan	Trp	40.0	Cystathionine II	39.7
Methionine	Met	40.5		
Valine	Val	41.4		
Phenylalanine	Phe	42.8		
Isoleucine	Ile	46.0		
Leucine	Leu	47.1	Hydroxylysine	47.7
Ornithine	Orn	49.8		
Lysine	Lys	50.6		

[a] The peak at 24.3 min arises from decomposition of methionine in plasma, and is observed when the concentration of methionine is very high.

In Table I we have listed amino acids usually found in our plasma samples and their retention times in order to indicate possible interferences.[a]

The Nova-Pak cartridges, unlike the Resolve cartridges, have been extremely durable, and retention and selectivity characteristics do not change appreciably over several hundred injections. Preliminary data have been presented for alcoholic hepatitis subjects from a large, multifaceted, cooperative Veterans Administration study [9] and will be published in detail after statistical analyses have been done.

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REFERENCES

1 D. Hogan, K. Kraemer and J. Isenberg, *Anal. Biochem.*, 127 (1982) 17.
2 D. Turnell and J. Cooper, *Clin. Chem.*, 28 (1982) 527.
3 M. Fernstrom and J. Fernstrom, *Life Sci.*, 29 (1981) 2119.
4 B. Jones, S. Pääbe and S. Stein, *J. Liq. Chromatogr.*, 4 (1981) 565.
5 J. Schmidt and C. J. McClain, *J. Chromatogr.*, 419 (1987) 1.
6 J. W. Dolan, D. C. Lommen and L. R. Snyder, *J. Chromatogr.*, 485 (1989) 91.
7 J. Schmidt, *J. Chromatogr.*, 485 (1989) 421.
8 J. Cooper and D. Turnell, *J. Chromatogr.*, 227 (1982) 158.
9 J. Schmidt, L. S. Marsano and C. J. McClain, *FASEB J.*, 4 (1990) A364.

[a] Resolution being critical for several bands, small changes in gradient and multi-solvent switch timing were required to accommodate individual variations in retentiveness and selectivity between different individual Nova-Pak cartridges. Therefore, some details in the scheme given in the Experimental section vary slightly from conditions used to acquire the data presented in the table and figure, which were acquired with different individual cartridges.