Journal of Chromatography, 634 (1993) 317-322
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 791

Automated preseparation derivatization on a capillary electrophoresis instrument

R.J.H. Houben, H. Gielen and Sj. van der Wal*

DSM Research, P.O. Box 18, 6160 MD Geleen (Netherlands)

(First received August 4th, 1992; revised manuscript received December 3rd, 1992)

ABSTRACT

Fully automated derivatixation prior to separation and the separation conditions were established for the determination of D-valine in an excess of L-valine by preseparation derivatixation with o-phthalaldehyde and N-acetylcysteine, separation of the resulting diastereomers by micellar electrokinetic capillary chromatography and absorbance detection. The precision and detection limit with absorbance detection are a factor of three worse than those achieved with HPLC and fluorimetric detection. The application of the method at high enantiomeric excess or with chemically impure samples is limited by the detector of the instrument.

INTRODUCTION

High-voltage capillary electrophoresis (CE) is a very efficient separation technique that suffers from a lack of detector sensitivity, but derivatization is a means of improving this. Postcolumn derivatization in CE has been shown to be feasible, but peak broadening in the reactor diminishes the efficiency benefit of CE [1]. Precolumn (preseparation) derivatization is an alternative that does not affect the efficiency.

In high-performance liquid chromatography (HPLC) the introduction of instrumentation for fully automated sample handling brought both the precision and the ease of use of precolumn derivatization within acceptable levels. This made precolumn derivatization in HPLC a popular technique and numerous suitable reagents for several functional groups are now available.

An analysis that is routinely performed in our laboratories by HPLC with automated precolumn derivatization is the determination of the enantiomeric purity of amino acids and their derivatives. The enantiomers are made to react with a chiral reagent and the resulting diastereomers are separated by reversed-phase HPLC. Several of these diastereomeric derivatives can be separated with equivalent resolution in a shorter time by micellar electrokinetic capillary chromatography (MECC) on a CE instrument [2]. Therefore, it can be expected that in CE the automation of preseparation derivatization will be at least as important as in HPLC. A prerequisite is that the instrumentation and reagents are generally available in sufficiently pure form and do not have to be assembled or synthesized, respectively.

The benefits of fully automated preseparation derivatization on a CE instrument are not limited to derivatization with chiral reagents or analyses of chiral compounds but stem from the increased precision and convenience of automation of the derivatization compared with manual operation on the one hand and the resolution that can be obtained when separating on a

* Corresponding author.

0021-9673/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

capillary system with voltage instead of pressure as a driving force on the other.

In this paper we focus, as an example, on the determination of D-valine in an excess of L-valine by preseparation derivatization, separation by MECC and absorbance detection.

EXPERIMENTAL

A P/ACE System 2050 (Beckman, Palo Alto, CA, USA) performed both the precolumn derivatizations and the separations and detection. To verify the concentrations used for the calibration graph of the P/ACE detector, the absorbance at 470 nm of solutions of potassium dichromate of different concentrations were measured in a l-mm cell on a Lambda 15 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). The calibration graph was then obtained, based on the assumption of a negligible effect of stray light for the lowest concentrations used.

The HPLC system used to determine the absorptivity of derivatives of several reagents at the wavelengths available with the P/ACE system, consisted of Gilson (Villiers-le-Bel, France) Model 305/302 pumps, a Rheodyne (Cotati, CA, USA) Model 7125 injector, a 50 x 4 mm I.D. Nucleosil 120-5-C_{18} column (Macherey-Nagel, Düren, Germany) and a Waters Model 990 diode-array detector (Millipore, Bedford, MA, USA). The eluent was a gradient from 10 to 90% acetonitrile in 10 m*M* phosphoric acid in 18 min at a flow-rate of 1 ml/min. L-Serine was employed as a test compound.

The reagents used in the absorptivity experiment were 1,2-phthalic dicarboxaldehyde (OPA) (Janssen, Beerse, Belgium) with N-acetylcysteine (NAC) (Janssen) [3] or N-acetyl-D-penicillamine (NAP) (Fluka, Buchs, Switzerland) [4] as a chiral thiol, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (Polysciences, Warrington, PA, USA) [5], 1-fluoro-2,4-dinitrophenyl-5-D-alanineamide (Marfey's reagent) (Pierce, Rockford, IL, USA) [2] and (+)-1-(9-fluorenyl)ethyl chloroformate (Flec) (Fluka) [6]. Each reagent was added sub-stoichiometrically to prevent interference from unreacted reagent.

The sample solution consisted of the indicated concentration of (total) amino acid in 0.12 *M* boric acid adjusted to pH 9.4 with 1 *M* sodium hydroxide solution.

The reagent solution was made by addition of 14 mg of OPA and 17 mg of NAC to a solution of 17% (v/v) ethanol and 83% aqueous 0.12 *M* boric acid. This reagent solution was adjusted to pH 9.4 with 1 *M* sodium hydroxide solution.

All other chemicals were of analytical-reagent grade (Merck, Darmstadt, Germany). All water was filtered through a Milli-Q apparatus (Millipore) and all solutions were filtered through a 0.22-μm nylon filter before use.

The eluting buffer for MECC was prepared by dissolving 0.1% (w/w) methylhydroxyethylcellulose 30 000 (MEC) (Serva, Heidelberg, Germany) and sodium dodecyl sulphate (SDS) to a 0.1 *M* concentration in 0.58 *M* acetic acid and adjusting the pH to 3.7 with 1 *M* tris(hydroxymethyl)aminomethane (Tris) (USB, Cleveland, OH, USA).

Owing to the high limit of detection with the UV detector, a high concentration of analyte has to be used, which necessitates a high buffer capacity during the derivatization, which in turn demands a high ionic strength of the separation buffer. The low mobility of Tris keeps the conductivity-to-buffer-capacity ratio of the eluent to a minimum. The peak symmetry and selectivity for the OPA-NAC derivatives is excellent at pH 3.7. A benefit of using 0.1% MEC and buffering at pH 3.7 is the stability of the retention times: the electroendosmotic flow is suppressed and the (negative) electrophoretic mobility of the derivatives dictates the retention.

Each new capillary was conditioned by a 10-min rinse with 1 *M* sodium hydroxide solution followed by several elongated blank runs. When the retention times of the derivatives do not decrease in consecutive runs the capillary is ready for use (if the electroendosmotic flow is still too large, no peaks may be detected at all).

RESULTS AND DISCUSSION

Detector linearity

The sensitivity of an absorbance detector is approximately proportional to the path length of the incident radiation in the detector. In CE this

path length is the diameter of the separation capillary, which is about 1% of the path length in a standard HPLC detector. This limits the dynamic range on the low absorbance side; stray light is the limiting parameter in the high absorbance region.

The percentage of stray light, S, can be calculated with the equation

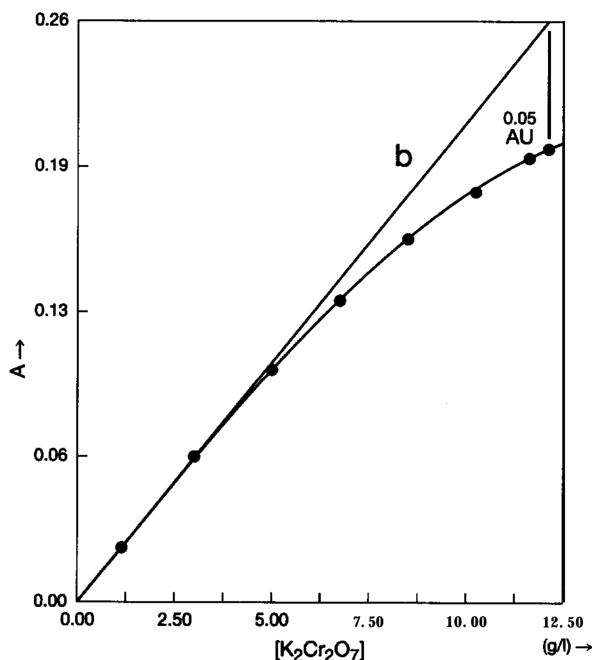

Fig. 1. Calibration graph to test the linearity of the absorbance detector. (b) Graph without stray light; (●) other experimental data.

$$\log\left(\frac{P_0}{P'}\right) = \log\left[\frac{1+S}{(P/P_0)+S}\right]$$

where P_0 = power of the incident beam, P = power of the exit beam, without stray light, and P' = power of the exit beam.

A calibration graph with potassium dichromate was obtained using the P/ACE system, a 50 μm I.D. capillary and a detector wavelength of 340 nm (Fig. 1). About 18% of stray light is present and correction for non-linearity is necessary above 0.1 absorbance. (The wide absorbance bands of potassium dichromate make the effect of band width on the non-linearity of the detector negligible.)

Derivatization reagent

An increasing number of chiral reagents are commercially available. To make a selection we determined the absorptivity at five wavelengths of five reagents for derivatization of (primary) amines that are available to us. The results are given in Table I. Several derivatives show considerable absorptivity at 214 nm; unfortunately, possible interferences are likely to absorb in this low-UV region also. Marfey's reagent has the most favourable spectral characteristics. Moreover, this reagent has been shown to result in exceptionally good enantioselectivities on MECC separation systems [2]. However, with the present P/ACE system the implementation of the automated derivatization with Marfey's reagent requires elevated temperature or very lengthy derivatization times, so to demonstrate the feasibility of precolumn derivatization the fast

TABLE I

ABSORPTIVITY OF DIFFERENT DERIVATIVES

Reagent	Molar absorptivity $\times 10^{-3}$ (1 mol^{-1} cm-‘)				
	200 nm	214 mn	254 nm	280 nm	340mn
OPA-NAC	23.7	31.7	10.7	4.0	10.6
OPA-NAP	26	31.5	13.3	4.7	13.3
GITC	17	7.7	3.8	1.6	0.5
Marfey's reagent	19	10	5.7	5.7	25
Flec	38	36	16	9.5	0

reaction of D,L-valine with OPA-NAC was chosen [3,7].

Reaction conditions

The full programme used for automatic derivatization and separation is given in Table II.

First a capillary volume of sample is taken (step 3), analogous to the precise method of complete loop filling in HPLC [8]. Then a timed amount of reagent is flushed backwards at fixed pressure together with the sample into a microvial (step 5) and a stream of nitrogen is used to mix the contents of the microvial during the reaction (step 7). The electrophoresis buffer is introduced into the separation capillary (step 9), injection takes place (step 10) and in addition to the separation (step 11) the capillary is cleaned to prevent memory effects (step 12). In between several steps carryover is eliminated by a dip in water of the possibly contaminated capillary end. The vial caps are constructed so that dilution by liquid adhering to the outside of the capillary is prevented.

The yield of the derivatization reaction is dependent on the pH and the excess of reagent in the microvial. Therefore, in addition to the reagent the sample solutions were buffered at the optimum pH of 9.4. At a reagent to sample ratio of 6.0-6.7 the excess of reagent has no significant influence on the result.

TABLE II

PROGRAM FOR AUTOMATED DERIVATIZATION FOLLOWED BY SEPARATION OF THE DERIVATIVES

Inlet vial[a]	Contents	Outlet vial	Contents
11	Separation buffer	1	Separation buffer
12-x	Analyte	2	Water
(x + 1)-31	Empty microvial	3	Reagent
33	Water	4	Water
34	Water	8	Empty
		10	Empty

Display Channel A with grid lines
Time: 0.00 to 10.00 Minutes
Channel A: -0.005 to 0.040 Absorbance

STEP	PROCESS	DURATION	INLET	OUTLET	CONTROL SUMMARY
1	SET DETECTOR				UV: 340 nm Rats: 10 Hz Normal Rise Range: 0200 -10% Zero 2.0 min.
2	SET TEMP				Tap: 25 °C
3	RINSE	2.0 min	12	8	Forward: High Pressure
4	WAIT	0.0 min	33	2	
5	RINSE	1.5 min	22	3	Reverse
6	WAIT	0.0 Pi"	22	4	
7	RINSE	1.0 min	22	10	Reverse
8	WI T	0.0 min	34	10	
9	RINSE	1.9 min	11	10	Forward: High Pressure
10	INJECT	25.01	22	10	Pressure
11	SEPARATE	10.0 min	11	1	constant Voltage: 30.00 kV Currant Limit: 100.0 "A Integrator On/Off
12	RINSE	3.5 min	11	10	Forward: High Pressure

[a] $x = 21$ for single analysis of ten samples; $x = 16$ for triplicate analysis of five samples, etc.

Quantitative analysis

The method of Table II was investigated for the determination of the enantiomeric composition of pure valine at a high enantiomeric excess of L-valine using external standard methods. When the chemical purity of the analyte is known, only the peak of the n-valine derivative has to be measured, which can be done at good signal-to-noise ratios using concentrations of the major derivative that may overload the phase system.

Four different concentrations of n-valine in an approximately constant total valine concentration were analysed in quadruplicate (see Table III and Fig. 2). The calibration graph indicates that the percentage of n-valine in this range can be determined with a 0.1% precision (95%

TABLE III

CALIBRATION GRAPH FOR D-VALINE

Experiment No.	Area of D-valine peak*			
	A	B	C	D
1	20.88	33.65	71.93	106.52
2	19.50	33.22	74.12	107.61
3	17.17	39.23	72.62	106.80
4	19.60	38.71	71.61	

[a] Total valine concentration and D-valine to L-valine ratio: (A) 36.0 mM and 0.0050; (B) 35.9 mM and 0.0097; (C) 35.9 mM and 0.0191; (D) 36.7 mM and 0.0283.

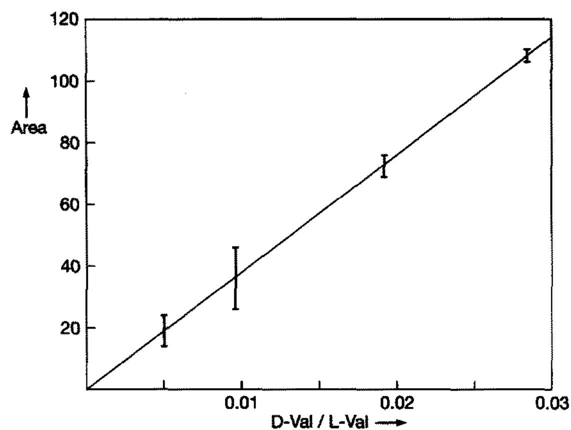

Fig. 2, Calibration graph for D-valine in excess of L-valine. Data are shown at the 95% confidence interval.

confidence interval) and a limit of detection of 0.3% D-valine in L-valine. A simpler one-point calibration gives a relative standard deviation (R.S.D.) of 6% at the 1% D-valine in L-valine level.

Improvement of the method

The precision and detection limit with absorbance detection obtained here are a factor of three worse than those achieved with HPLC and fluorimetric detection [7]. The precision could be improved by using the D/L ratio of the analyte. The ratio method also allows chemically impure analytes to be analysed. The validity of the improvement by the ratio method was tested on a racemic mixture (see Table IV). It is clear that this is a significant advance.

At a concentration of 34 m*M* L-valine elutes under overload conditions: the local pH is not well defined and the analyte peak is distorted and absorbs in the non-linear range of the detector (see Fig. 3A). To eliminate the distortion the concentration must be decreased to 13 m*M* (Fig, 3B), which gives an unacceptable increase in the limit of detection with the detector used.

A different buffer during the reaction with less capacity can give some extension of the linearity of the calibration graph at high analyte concentrations (e.g., by detection of the main component in a less sensitive way at a different wavelength than the trace component). While this is a minor improvement, a laser-induced fluorescence detector is likely to offer the sensitivity needed, with additional selectivity [9]. Such a detector with a helium-cadmium laser is being constructed in our laboratory.

Other means of improving the precision such as standard additions or reagent addition by capillary volume instead of a tuned constant pressure can be investigated when this detector is installed.

The promising results of this investigation (i.e., the better resolution and a shorter separation time with MECC compared with HPLC) in

TABLE IV

COMPARISON OF AREA AND AREA RATIO FOR PRECISION OF RESULTS

Experiment No.	Area 1	Area 2	Area 1/area 2	Retention time (s)	
				t_{r1}	t_{r2}
1	302.698	322.966	0.9372	6.649	7.075
2	298.328	321.499	0.9279	6.551	6.968
3	306.909	321.108	0.9558	6.570	4.989
4	298.574	320.887	0.9305	6.572	6.992
5	307.495	326.802	0.9409	6.575	6.998
6	278.609	297.657	0.9360	6.592	7.018
7	302.348	319.821	0.9454	6.600	7.027
8	318.416	340.876	0.9341	6.589	7.020
9	298.680	314.274	0.9504	6.615	7.049
10	309.527	330.771	0.9358	6.609	7.044
R.S.D. (%)	3.4	3.5	1.0	0.4	0.5

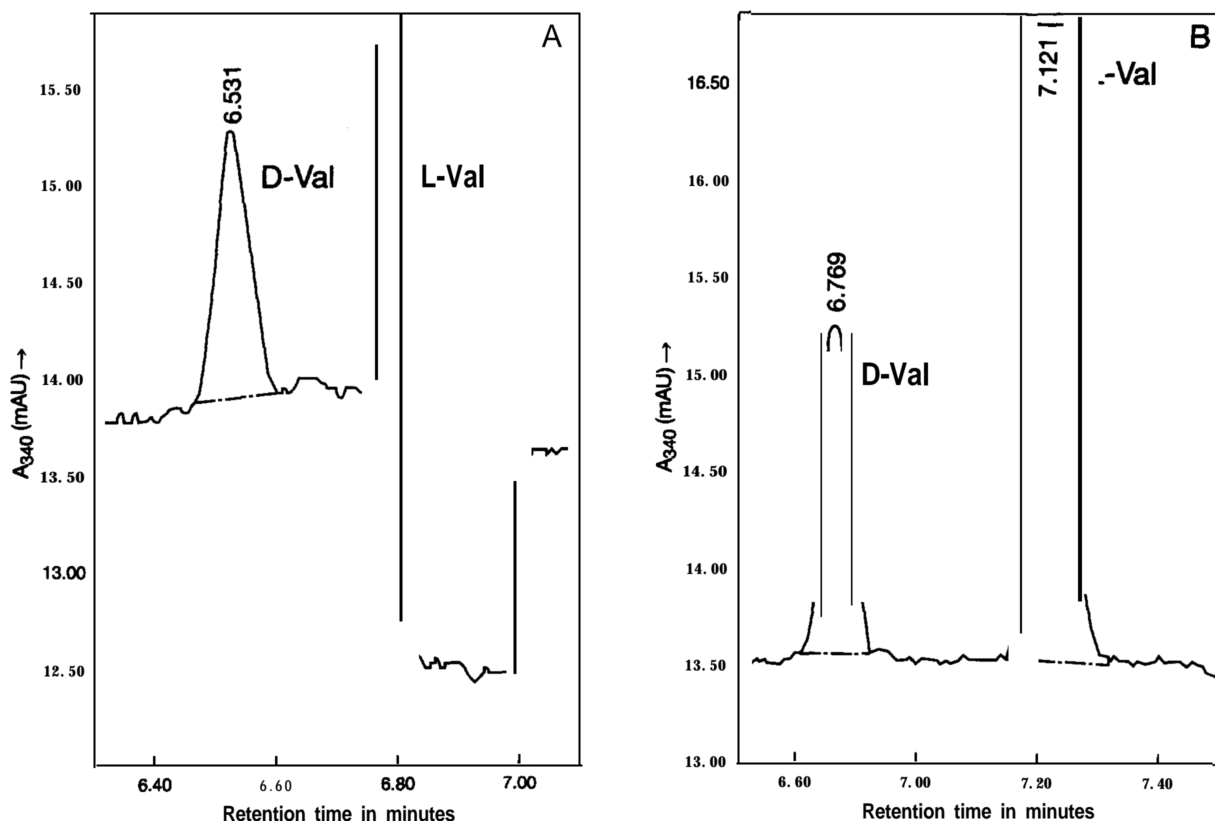

Fig. 3. Chromatogram of D,L-valine derivatives at the (A) 34 m*M* and (B) 13 m*M* levels.

our opinion warrant the research effort and increased instrumental expense. With the instrument manufacturers being aware of these developments, even more versatile software will become available to suit user-specific applications .

ACKNOWLEDGEMENT

We appreciate the opportunity given by the Higher Institutes for Professional Education at Heerlen and Venlo to their students R.J.H.H. and H.G. to complete their graduation work at DSM Research.

REFERENCES

1 D.J. Rose and J.W. Jorgenson, *J. Chromutogr.*, 447 (1988) 117.

2 A.D. Tran, T. Blanc and E.J. Leopold, *J. Chromutogr.*, 516 (1990) 241.

3 R.H. Buck and K. Krummen, *J. Chromatogr., 387 (1984) 279.*

4 R.H. Buck and K. Krummen, *J. Chromutogr., 387 (1987) 255.*

5 A. Jegorov, V. Matha, T. Tmka and M. Cerpy, *J. High Resolut. Chromatogr., 13* (1990) 718.

6 S. Einarsson and B. Josefsson, *Anal. Chem.*, 59 (1987) 1191.

7 A. Duchateau, M. Crombach, J. Kamphuis, W.H.J. Boesten, H.E. Schoemaker and E.M. Meijer, *J. Chromutogr., 471 (1989) 263.*

8 *Technical Note No. 5,* Rheodyne, Cotati, CA, December 1983.

9 B. Nickerson and J.W. Jorgenson, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 878.