Journal of Chromatography, 213 (1981) 327–330
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,910

Note

Resolution of enantiomers of norepinephrine and epinephrine by reversed-phase high-performance liquid chromatography

NORIYUKI NIMURA*, YOKO KASAHARA and TOSHIO KINOSHITA
School of Pharmaceutical Sciences, Kitasato University, 9-1 Shirokane-5, Minato-ku, Tokyo 108 (Japan)
(Received April 21st, 1981)

Catecholamines such as norepinephrine and epinephrine are important substances in the fields of biological and clinical chemistry. We have recently reported[1] a simple fluorimetric determination of catecholamines by high-performance liquid chromatography (HPLC). The chromatographic resolution of catecholamine enantiomers, which is important for the elucidation of their biological conversion[2], is dealt with in the present study.

In the previous paper[3], reversed-phase HPLC resolutions of amino acid enantiomers by pre-column chiral derivatization with acetylglycosyl isothiocyanates, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC), were described.

This paper describes the liquid chromatographic resolution of D,L-norepinephrine and D,L-epinephrine on a reversed-phase column using GITC and AITC. Diastereomeric thiourea derivatives which were formed from catecholamines with either GITC or AITC could be resolved on an octadecylsilyl-bonded silica gel column with methanol–10 m*M* phosphate buffer (pH 2.8) as a mobile phase, and detected spectrophotometrically at 250 nm. Excellent resolution was observed without protection of phenolic hydroxyl groups and the procedure was very simple.

MATERIALS AND METHODS

Racemic and L-isomeric norepinephrine and epinephrine were obtained from Sigma (St. Louis, MO, U.S.A.). Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Chemical Industry Company (Tokyo, Japan). All the reagents were of analytical reagent grade. Methanol and water were distilled before use. GITC and AITC were prepared by treatment of α-acetobromoglucose and β-acetobromoarabinose with silver thiocyanate, as described previously[4]; GITC and AITC are commercially available from Polysciences (Warrington, PA, U.S.A.). The 10 m*M* phosphate buffer was prepared from monobasic potassium phosphate and was adjusted to pH 2.8 with perchloric acid.

Equipment

The chromatographic system consisted of a high-pressure pump equipped with a valve universal injector (Sanuki Industry Company, Tokyo, Japan), a Develosil

0021-9673/81/0000–0000/S02.50

ODS column (15 cm × 4.6 mm I.D., particle size 5 μm; Nomura Chemical, Seto-shi, Japan), and an SPD-2A spectrophotometric detector (Shimadzu Seisakusho, Kyoto, Japan).

Derivatization and separation procedure

Each 10 mg of racemic catecholamine and each 5 mg of L-isomer were dissolved in 0.25 *M* aqueous acetic acid to make 100 ml, respectively. Then 50 μl of this catecholamine stock solution was pipetted into a microtube, and evaporated to dryness under reduced pressure in a desiccator at room temperature. To the residue was added 50 μl of 0.2% (w/v) chiral reagent, either GITC or AITC, in dimethylformamide (DMF). This reaction mixture was allowed to stand at room temperature for 10 min, and then 10 μl of 0.5% (v/v) hydrazine hydrate DMF solution were added. The resulting mixture was allowed to stand at room temperature for 10 min, and then a 10-μl aliquot of the mixture was injected directly into the chromatograph. The column was eluted at room temperature and at a flow-rate of 0.9 ml/min, with a mobile phase prepared by mixing methanol and 10 m*M* phosphate buffer, pH 2.8, in an appropriate ratio.

RESULTS AND DISCUSSION

Norepinephrine and epinephrine react readily with chiral reagents, either GITC or AITC, under mild conditions without the formation of by-products. The resulting mixture can directly be injected into the chromatograph. The thiourea derivatives eluted from the column were monitored using the absorption at 250 nm[3,4].

Fig. 1 shows the amount of thiourea derivatives formed from L-norepinephrine with AITC *versus* the reaction time for the two different reaction solvents: DMF and acetonitrile. The amount of thiourea formed is proportional to the peak height. With DMF as the reaction solvent, the reaction proceeded to completion in 10 min. In contrast, more than 50 min were needed when acetonitrile was used, and the yield of

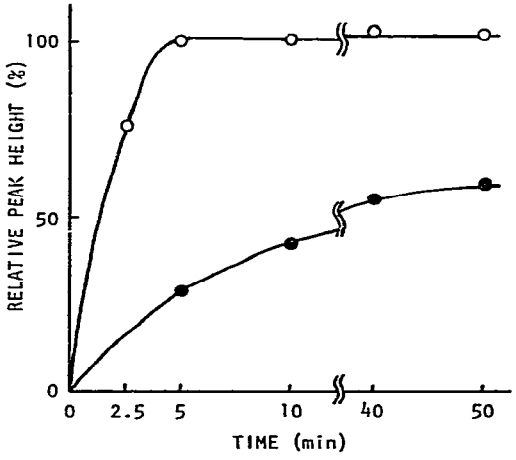
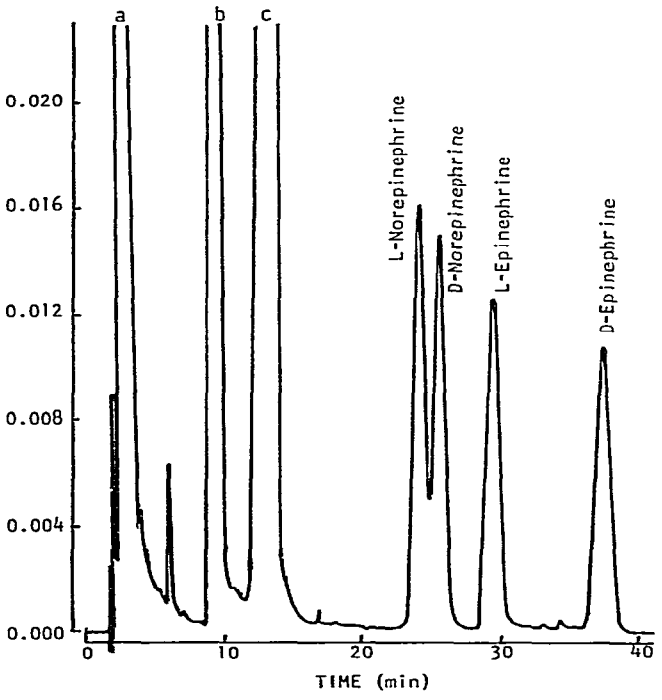


Fig. 1. Dependence of the reaction yield of the thiourea derivative formed from L-norepinephrine with AITC. ○, in DMF; ●, in acetonitrile.

the reaction was low. Recently, Björkqvist[5] reported that the DMF both seemed to catalyse the reaction and also served as a good solvent for the disubstituted urea, in his study of the derivatization of aliphatic and aromatic amines using phenylisocyanate.

Figs. 2 and 3 show the chromatograms of the diastereomeric thiourea derivatives of isomeric norepinephrine and epinephrine when GITC and AITC were used for the derivatization, respectively. Enantiomeric pairs were eluted in the sequence L before D when GITC was used, but in the opposite sequence when AITC was used. These results are in good agreement with those of enantiomeric amino acids[3].



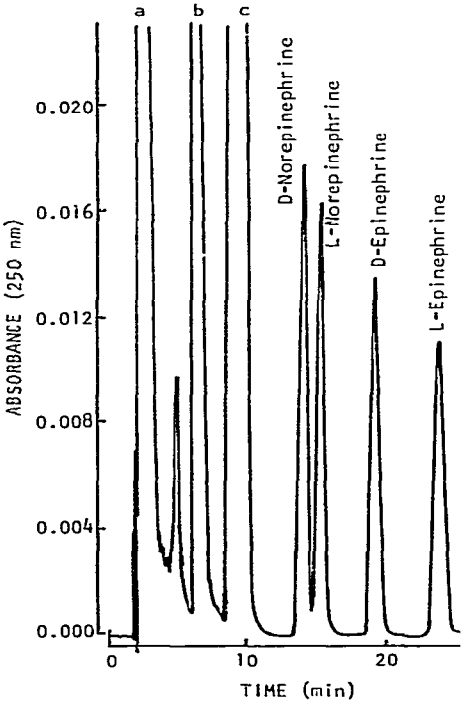


Fig. 2. Separation of diastereomeric thiourea derivatives formed from catecholamines with GITC. Mobile phase, methanol–10 m*M* phosphate buffer (pH 2.8) (35:65). Flow-rate, 0.9 ml/min. About 200 ng of each derivative were injected. Peaks: a = DMF; b, c = reaction products of the excess reagent with hydrazine.

Fig. 3. Separation of diastereomeric thiourea derivatives formed from catecholamines with AITC. Mobile phases, methanol–10 m*M* phosphate buffer (pH 2.8) (30:70). Flow-rate, 0.9 ml/min. About 200 ng of each derivative were injected. Peaks: a = DMF; b, c = reaction products of the excess reagent with hydrazine.

Under the same chromatographic conditions, the reagent peaks are well separated from those of the diastereomers and do not interfere with the detection. However, when the injection of samples is repeated, peaks of the excess reagents interfere with the analysis. Consequently, hydrazine hydrate was added to the derivatization mixture to remove of excess reagents. Hydrazine reacted completely with the excess reagents and the reaction products were eluted faster than any diastereomers (Figs. 2 and 3).

The retentions and resolutions of the diastereomeric GITC and AITC derivatives are listed in Table I; k', α and R_s refer to the capacity ratio, separation factor and resolution respectively for a pair of diastereomers. The resolution of AITC derivatives were favored over that of GITC derivatives.

TABLE I

SEPARATION OF DIASTEREOMERIC THIOUREA DERIVATIVES FORMED FROM CATECHOLAMINES WITH GITC AND AITC

t_0 = 2.0 min. Column, Develosil ODS (15 cm × 4.6 mm I.D.). Mobile phase: methanol–10 m*M* phosphate buffer (pH 2.8) (35:65) (A), (30:70) (B).Flow-rate, 0.9 ml/min. k', α and R_s are defined in the text.

Catecholamine	*GITC*				*AITC*			
	k'	α	R_s	*Mobile phase*	k'	α	R_s	*Mobile phase*
Norepinephrine D	11.40	1.08	1.00	A	6.00	1.10	1.20	B
L	10.60				6.60			
Epinephrine D	17.40	1.30	4.00	A	8.60	1.28	3.69	B
L	13.40				11.00			

Nambara and co-workers[6,7] pointed out that the rigidity of the conformation around the chiral centres, and the proximity between the chiral centres of the diastereomer, are important to secure satisfactory resolution. They have achieved good resolution of amino acid enantiomers using terpene isothiocyanate reagents. However, this reagent required the conversion of the amino acid moiety into the bulky *tert.*-butyldimethylsilyl ester. GITC and AITC facilitated excellent resolution of amino acids without esterification of carboxyl groups[3]. This fact may be attributed to the sterically crowded acetylglycosyl residues of the reagents, which may increase the conformational rigidity. Chiral centres of the catecholamines are located at the β-position from their amino groups, and the distances between the chiral centres of their GITC or AITC derivatives are therefore greater than those of amino acid derivatives. Nevertheless, the catecholamine derivatives were well resolved, presumably owing to the bulkiness of GITC and AITC residues. These bulky groups may fix the conformation around the asymmetric carbon of the catecholamines.

In addition, the bulky hydrophobic group of the present reagent seemed to favour resolution on the ordinary reversed-phase column.

The present method may generally be applicable to the resolution of optical isomers whose chiral centres are too distant from the functional groups to react with the chiral reagents.

REFERENCES

1 N. Nimura, K. Ishida and T. Kinoshita, *J. Chromatogr.*, 221 (1980) 249.
2 T. Nagatsu, *Biochemistry of Catecholamines*, University of Tokyo Press, Tokyo, and University Park Press, Baltimore, MD, 1973, pp. 1–362.
3 T. Kinoshita, Y. Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
4 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
5 B. Björkqvist, *J. Chromatogr.*, 204 (1981) 109.
6 T. Nambara, S. Ikegawa, M. Hasegawa and J. Goto, *Anal. Chim. Acta*, 101 (1978) 111.
7 J. Goto, M. Hasegawa, S. Nakamura, K. Shimada and T. Nambara, *J. Chromatogr.*, 152 (1978) 413.