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Chromatographic determination of enantiomeric purity by achiral means

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ABSTRACT

A chromatographic method for determination of the enantiopurity of a chiral analyte using achiral reagents is described. The analyte is first converted to diastereomers utilizing either dimerization reactions of the analyte or a bifunctional (achiral) reagent. The diastereomers thus obtained constitute a *meso* compound and a *dl* pair, *e.g.* two epimeric pairs. The epimeric pairs are separated by HPLC on a C_{18} (achiral) column. A mathematical equation was derived for determination of enantiomeric purity utilising the peak areas of the eluted epimers. This method takes into account differences in both reaction rates of the formation of the epimers and differences in their detector responses. The minimum amount of enantiomeric impurities (in the analytes studied) that could be detected by this method was approximately 0.002%.

INTRODUCTION

Determination of the enantiomeric composition of an analyte is routinely made by either direct or indirect methods. Direct separation of enantiomers based on a chiral stationary phase (CSP) is, of course, the method of choice. CSPs, however, frequently show low efficiencies and/or loading capacities, which restrict their ability to determine very small amounts of optical impurities in the analytes. The enantiomeric purity of the analyte can be determined even when the chiral selector is partially racemized [1]. Chiral additives [2], not necessarily enantiomerically pure, in the mobile phase in combination with an achiral column are also commonly used in evaluation of the enantiomeric composition of analytes.

An indirect method is based on the preparation of diastereomers by reacting the solute with a chiral reagent followed by separation of the epimers on an achiral column. The limitation of this approach is the enantiomeric purity of the chiral reagent. Determination of enantiomeric contaminants less than, say, 0.1% requires a reagent with an enantiomeric purity of at least 99.9%.

Vingeron *et al.* [3] were the first to recognize the possibility of utilizing the intrinsic difference in chirality between a pure enantiomer and an enantiomeric mixture for determination of optical purity by means of bifunctional achiral reagents. By reacting enantiomers with a bifunctional symmetrical achiral reagent it was possible to convert the mixture of enantiomers into a *meso* compound and a *dl* pair. Vingeron *et al.* [3]

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showed that it is possible to determine the optical purity of the initial enantiomer by analysing the diastereomeric mixture [1] by either NMR or gas chromatography. A similar method utilizes phosphorus trichloride (PCl_3) as derivatizing agent and the integrals of ^{31}P NMR signals for determination of enantiomeric purity [4]. A somewhat modified version of this ^{31}P NMR [5] method allows determination of enantiomeric excess within 2%, the limitation being the accuracy of the integration due to the signal-to-noise ratio.

The potential of the method of Vingeron *et al.* [3] has further been demonstrated using ligand exchange chromatography [6] or gas chromatography [7] to separate the *meso* compound and the *dl* pair. We report a method based on the original approach of Vingeron *et al.* [3] in which the diastereomers are prepared by randomly linking the enantiomers of an analyte either directly by a disulphide bridge or by means of an achiral bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene (DFDNB). The diastereomers formed are separated by HPLC on a C_{18} phase using an achiral mobile phase.

This report also includes the derivation of a mathematical equation for the calculation of the enantiomeric purity as the peak areas of the eluted epimers are not directly proportional to the concentrations of the initial enantiomers of the analyte. In the derivation of the mathematical expression differences in reaction due to chiral discrimination in the formation of the diastereomers as well as differences in their detector response factors are considered.

The accuracy of the method was tested by determination of the enantiomeric composition of two different kinds of chiral analytes, one

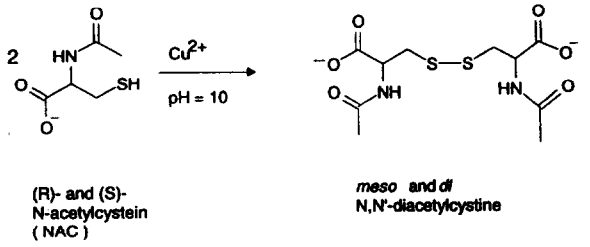

Fig. 1. Synthesis of *meso* and *dl* N,N'-diacetylcysteine.

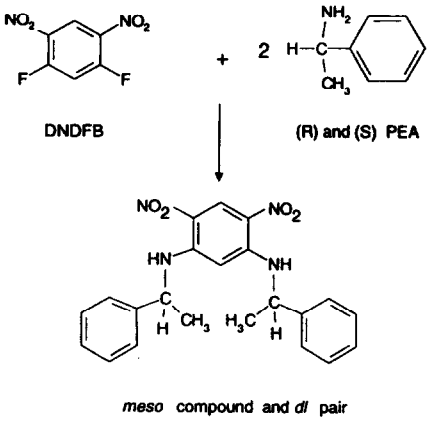

Fig. 2. Coupling of (*R*)- and (*S*)-PEA to bifunctional reagent DNDFB.

containing mixtures of (*R*)- and (*S*)-N-acetylcysteine (NAC) and the other containing mixtures of (*R*)- and (*S*)-1-phenylethylamine (PEA). The enantiomers of both samples were converted to diastereomers, a *meso* compound and a *dl* pair, by achiral means (see Figs. 1 and 2). The epimers were in a subsequent step separated by HPLC using a C_{18} phase (see Figs. 3 and 4). To allow comparisons the enantiomeric composition of PEA was also determined using a chiral reagent (Marfey's reagent [8]). The main

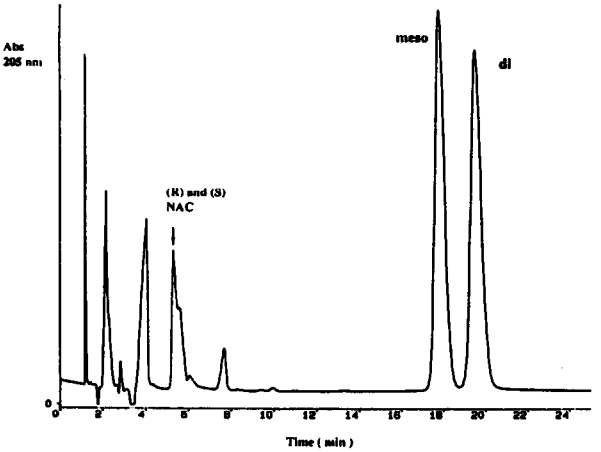

Fig. 3. Separation of *meso* and *dl* N,N'-diacetylcystine epimers by HPLC. Column: Kromasil DR 100-5 C_{18}, 150 × 4.6 mm I.D. Mobile phase: (20 m*M* tetrabutylammonium sulphate + 10 m*M* NaH_2PO_4, pH 7.0)–acetonitrile (95:5, v/v). Detector: UV 205 nm. Flow-rate: 1.0 ml/min. Injection volume: 20 μl.

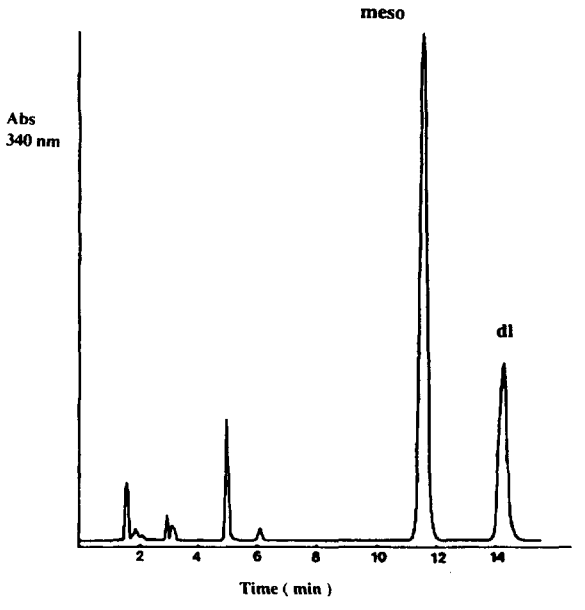

Fig. 4. HPLC separation of *meso* and *dl* PEA. Column: Supelcosil LC-C_{18}, 5 μm, 150 × 4.6 mm I.D. Mobile phase: 10 m*M* NaH_2PO_4, pH 7.0–acetonitrile (30:70, v/v). Detector: UV 340 nm. Flow-rate: 1.0 ml/min. Injection volume: 20 μl.

purpose of this work was to get access to highly purified PEA. The enantiopurity of the material was determined after each recrystallization according to the method presented in this paper.

EXPERIMENTAL

Apparatus and chromatography

A Hewlett-Packard 1050 (Hewlett-Packard, Waldbronn, Germany) was used as solvent-delivery system and a Kratos Spectroflow 783 (ABI Analytical, Ramsey, NJ, USA) as detector. The analytes were introduced onto the column by a Carnegie Medecin CMA/200 autoinjector (Carnegie Medecin, Stockholm, Sweden). The chromatographic conditions are described in the figure legends.

The peak areas were calculated using a chromatographic data system (Nelson Analytical, Cupertino, CA, USA).

Chemicals and reagents

DFDNB was obtained from Jansen (Beerse, Belgium). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) was supplied by Pierce (Rockford, IL, USA). (*R*)- and (*S*)-PEA were purchased from Merck (Darmstadt, Germany) and NAC was prepared (*i.e.* acetylation of cysteine) at Draco (Lund, Sweden).

Purification of (R) or (S)-1-phenylethylamine

To remove possible enantiomeric impurities in the commercial (*R*)- and (*S*)-(enantiomers) PEA, these were recrystallized as diastereomeric salts according to a previously published method [9]. A 4.0-g aliquot of (*R*) or (*S*)-PEA and 5.0 g of (−)- or (+)-tartaric acid were dissolved in 15 ml of boiling water and the solution was allowed to crystallize overnight. The isolation of the enantiomer after each recrystallization was carried out as previously described. All other chemicals were of analytic or chromatographic grade and used as obtained.

Preparation of diastereomeric derivatives

N,N'-Diacetylcystine. NAC is converted to N,N'-diacetylcystine according to a standard procedure (see Fig. 1). To approximately 1.0 g of NAC dissolved in a 0.2 *M* sodium carbonate buffer (pH 10) 50 μl of 8.0 m*M* copper sulphate solution were added. The reaction mixture was allowed to stand for 3–10 h, after which time 20 μl of the crude mixture were injected onto the column.

Preparation of diastereomers of PEA with DFDNB. PEA was allowed to react with DFDNB according to Fig. 2. A 200-μl sample of a 10.0 m*M* solution of PEA (as free base or salt) in water, 400 μl of a 2.5 m*M* solution of DFDNB in acetone and 80 μl of 1.0 *M* sodium carbonate buffer (pH 9.4) were mixed and kept at 40°C. After 1 h the mixture was acidified with 80 μl of 2 *M* HCl. A 20-μl aliquot of this mixture was injected onto the column.

Occasionally a small amount of precipitated material was observed, which could change the diastereomeric ratio of the solution. This could be avoided by exchanging the sodium carbonate buffer for 10 μl of triethylamine.

Preparation of diastereomers of PEA with Marfey's reagent. Marfey's reagent, 2.5 m*M* acetone, was used according to the same procedure as DFDNB.

DERIVATION OF EQUATIONS

Formation of diastereomers from a mixture of enantiomers could be accomplished either by a direct dimerization or by means of a bifunctional reagent (A). In the following derivations the bifunctional reagent is included but the resulting equations are unaffected whether or not a reagent is used.

Reacting a bifunctional symmetrical achiral reagent, A, with enantiomers of an analyte, (*R*) and (*S*), gives the *dl* pair (*S*)-A-(*S*) and (*R*)-A-(*R*) and the *meso* compound (*S*)-A-(*R*) or (*R*)-A-(*S*) (see Fig. 5). The (*S*)-A-(*R*) and (*R*)-A-(*S*) compounds are identical due to the symmetrical reagent, A.

The *meso* and *dl* compounds are epimers and can be separated by achiral means. In the first stage of the reaction the enantiomers, (*R*) and (*S*) In Fig. 5, with molar ratios x and $1-x$, respectively, yield the intermediates (*R*)-A and (*S*)-A with molar ratios x and $1-x$, respectively. In the next stage of the reaction the intermediates (*R*)-A and (*S*)-A can react with either (*R*) or (*S*) to form (*R*)-A-(*R*) or (*R*)-A-(*S*) and (*S*)-A-(*R*) or (*S*)-A-(*S*), respectively. However, it is likely that the epimers (*R*)-A-(*R*) and (*R*)-A-(*S*) are formed at different reaction rates and consequently the amount of (*R*)-A-(*R*) and (*R*)-A-(*S*) that is formed will be proportional to xxk_2 and $(1-x)xk_1$, respectively, where k_2 and k_1 are constants that take into account different reaction rates and/or different detector response factors.

It can be assumed that the enantiomers (*R*)-A-(*R*) and (*S*)-A-(*S*) are formed at the same reaction rate and have the same UV response.

The area of the peak containing the *meso* compound (Q_1) and the area of the peak containing the *dl* pair (Q_2) are related to the original amounts of (*R*)- and (*S*)-enantiomers in the analyte in the following manner.

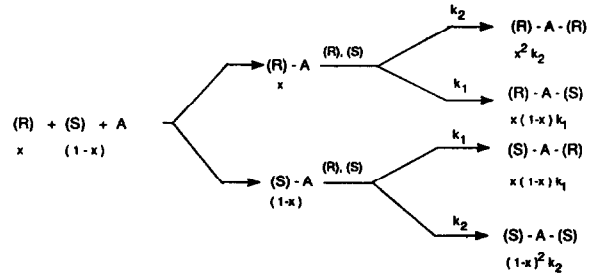

Fig. 5. Symbols used in the derivation of the equations.

$$\frac{Q_1}{Q_1+Q_2} = q_1 = \frac{2k_1x(1-x)}{2k_1x(1-x)+k_2x^2+k_2(1-x)^2}$$

If $K = \frac{k_2}{k_1}$

$$q_1 = \frac{2x(1-x)}{2x(1-x)+Kx^2+K(1-x)^2} \quad (1)$$

Eqn. 1 gives x as a function of K and q_1:

$$x = 0.5 \pm \sqrt{0.25 - K/2(1/q_1 + K - 1)} \quad (2)$$

Each value of q_1 gives two possible solutions of x, which means that there are always two possible compositions (x and $1-x$) of the original enantiomeric mixture (analyte) that will give the same peak area ratio (q_1). Consequently, this method cannot be used to determine which of the enantiomers, (*R*) or (*S*), is predominant in a specific sample, but only their relative amounts.

To solve the equations, K has to be determined from a sample with known composition. The simplest way to do this is with a racemate where $x = 0.5$. Eqn. 1 then takes the simple form:

$$q_1 = \frac{1}{1+K} \text{ or}$$

$$K = \frac{1}{q_1} - 1 = \frac{Q_2}{Q_1} \quad (3)$$

If $K = 1$ then eqn. 2 is simplified to:

$$x = 0.5 \pm \sqrt{0.25 - 0.5q_1} \quad (4)$$

For very small values of x ($1-x = 1$, $x^2 = 0$ and $1-q_1 = 1$) eqn. 1 reduces to:

$$q_1 = \frac{x}{x+0.5K} \text{ or } x = 0.5q_1K$$

RESULTS AND DISCUSSION

In order to check the reliability of the equations above, a series of different enantiomeric mixtures of purified PEA with known compositions (x) were prepared. The mixtures were

analysed and q_1 was determined for each mixture. With an iterative regression program a value of K was calculated that made the best fit to eqn. 2 of all x and corresponding q_1 values. The enantiomeric purity of an analyte is then easily obtained from eqn. 2 using the calculated K value and q_1, which is obtained from the evaluation of the chromatogram. The results are shown in Table I. Each value of q_1 is the mean of at least two runs. The best fit to eqn. 2 was obtained with $K = 0.51927$, which shows that the reaction rate for formation of the *meso* compound is almost twice the reaction rate for the formation of the *dl* pair.

Marfey's reagent is well recognized for its usefulness as a chiral derivatizing reagent, especially for amino acids. It is structurally similar to DFDNB; one of the fluorine atoms is replaced by L-alanine amide. In another set of experiments the enantiomeric purity of PEA was determined with both Marfey's reagent and DFDNB after each recrystallization (see *Chemicals and reagents* section) in the purification of PEA. Each determination is a mean value of at least two chromatographic runs and the results are shown in Table II.

As the content of (*R*) in (*S*) decreases, the determinations performed with Marfey's reach a level of approximately $x = 0.0025$, in contrast to the determinations performed with DFDNB. This discrepancy is probably due to a contamination of Marfey's reagent with approximately 0.25% of the opposite enantiomer.

The surprisingly pure product obtained after the second recrystallization could be due to a non-representative sampling.

Enantiomeric mixtures of NAC of known compositions were also prepared and analysed, and the results are shown in Table III. In this case the reaction rates for the formation of the *meso* and the racemic forms are very similar ($K \approx 1$). A good correlation is obtained between added and found values.

TABLE I

DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF PEA IN MIXTURES OF KNOWN COMPOSITION

Molar ratio of (*R*) (added x)	Peak area ratio (q_1)	Molar ratio of (*R*) (found x)[a]
0.00016	0.00067	0.00017
0.00067	0.00272	0.00071
0.00304	0.0115	0.00301
0.0123	0.0461	0.0124
0.0355	0.1222	0.0349
0.1222	0.3455	0.1226

[a] Regression analysis: Minimum (the best fit) was found after 23 iterations. The *residual sum of squares* at minimum was $4.111 \cdot 10^{-6}$.

TABLE II

DETERMINATION OF ENANTIOMERIC PURITY USING MARFEY'S REAGENT OR DFDNB

Batch of PEA (number of recrystallizations)	Marfey's reagent [molar ratio of (*R*)]	DFDNB [molar ratio of (*R*)]
0[a]	0.00208	0.0156
1	0.0070	0.0040
2	0.0027	0.00019
3	0.0033	0.00052
4	0.0030	0.00042
5	0.0025	0.000034

[a] Starting material.

CONCLUSIONS

The success of the determination is highly dependent on a simple reaction between a

TABLE III

DETERMINATION OF ENANTIOMERIC COMPOSITION OF NAC IN MIXTURES OF KNOWN COMPOSITION

Molar ratio of (*R*) added (x)	Peak-area ratio (q_1)	Molar ratio of (*R*) found (x)
0	0.0003	0.00015
0.0053	0.0099	0.0050
0.0154	0.0285	0.0145
0.0538	0.1022	0.0542
0.1584	0.2591	0.1534
0.3090	0.4209	0.3022
0.5000	0.4991	0.5000

bifunctional reagent and substrate or a dimerization of the substrate.

The detection limit is mostly dependent on the linear dynamic range of the detector and the loading capacity of the column. Detection of enantiomeric impurity of PEA and DFDNB was possible down to approximately 0.002%. DFDNB has a strong UV absorption maximum at 340 nm, which is often distinguishable from the absorption of most organic compounds. This facilitates the identification of the reaction products. However, the method does not permit the identification of each enantiomer; only a determination of their relative concentrations is possible.

Detection of enantiomeric impurity of NAC by dimerization was possible down to at least 0.02%. In this case the native UV absorbance of N,N′-diacetylcystine at 205 nm was utilized for detection.

The great variety of commercially available bifunctional reagents should make it possible to find suitable ones that fulfil the requirements for derivatization and the subsequent chromatographic separation of a number of chiral compounds.

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REFERENCES

1 U. Beitler and B. Feibush, *J. Chromatogr.*, 123 (1976) 149–166.
2 C. Pettersson, A. Karlsson and C. Gioeli, *J. Chromatogr.*, 407 (1987) 217–229.
3 J.P. Vingeron, M. Dhaenens and A. Horeau, *Tetrahedron*, 29 (1973) 1055.
4 B.L. Feringa, A. Smaardijk and H. Wynberg, *J. Am. Chem. Soc.*, 107 (1985) 4798.
5 C.J. Welch, *Tetrahedron: Asymmetry*, 2 (1991) 1127.
6 Yu.P. Belov, A.P. Sidorov and I.V. Martynov, *Dokl. Akad. Nauk SSSR*, 308 (1989) 253–274; *Dokl. Chem. (Engl. Transl.)*, 308 (1990) 272–273.
7 I.N. Stankov, S.N. Tarasov, V.V. Lysenko, A.N. Beresnav, K.I. Sakodynskii and F.E. Shatilo, *Zh. Fiz. Khim.*, 64 (1990) 2467.
8 S.P. Marfey and K.H. Tsai, *Biochem. Biophys. Res. Commun.*, 65 (1975) 31–38.
9 A.H. Blatt, *Organic Synthesis*, Collective Volume 2, Wiley, New York, 1959, p. 506.