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# Enantiospecific drug analysis via the *ortho*-phthalaldehyde/homochiral thiol derivatization method*

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## ABSTRACT

Pre-column derivatization with *o*-phthaldialdehyde and an optically active thiol has hitherto been used mainly for liquid-chromatographic chiral separation of amino acids. Chiral separation of non-amino-acid primary amines, especially of *pharmaceuticals*, via this approach has been largely ignored. We have therefore examined the applicability of the method to the chiral resolution of several pharmaceutical amines. *o*-Phthaldialdehyde and four commercially available homochiral thiols were used to study the separation of the enantiomers of amphetamine, *p*-hydroxyamphetamine, *p*-chloroamphetamine, 3-amino-1-phenylbutane, 3-amino-1-(4-hydroxyphenyl)-butane, mexiletine, tocainide, tranylcypromine and rimantidine. The resulting highly fluorescent isoindole derivatives were resolved on a Waters Nova-Pak $C_{18}$ column using mobile phases consisting of mixtures of methanol, a sodium acetate buffer and acetonitrile, and the column effluent was monitored using fluorescence or UV detection. In some cases the fluorescence and/or the UV absorbance of the two diastereomers were unequal. It was found that the resolution of most of the amines could be optimized by varying the homochiral thiol in the derivatization step. This method of chiral separation may have wide applicability in enantiospecific drug analysis of non-amino-acid primary amines due to its simplicity and the high sensitivity it provides.

## INTRODUCTION

In 1971 Roth [1] described a sensitive analytical method for amino acids based on their reaction with *o*-phthaldialdehyde (OPA) and a thiol, 2-mercaptoethanol. The reaction produces an intensely fluorescent isoindole derivative of the amino acid and is specific for the primary amino group [2,3]. Early applications of this method used an amino acid analyzer in conjunction with postcolumn derivatization [1,4], but subsequently precolumn derivatization combined with high-performance liquid chromatography (HPLC) gained popularity [5–8].

More recently, the OPA method was extended to the enantiospecific liquid-chromatographic analysis of amino acids by substituting a homochiral (single enantiomer) thiol for 2-mercaptoethanol in the derivatization reaction [9–14]. This results in the formation of two diastereomeric isoindoles derived from the enantiomeric amino acids, respectively, and the derivatives are then separated on conventional (non-chiral) HPLC columns. This approach to enantiospecific chromatographic analysis, *i.e.*, derivatization with a homochiral reagent to form diastereomers prior to chromatography, is often termed the indirect approach because the enantiomers are not separated as such but as dia-

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stereomeric derivatives [15]. The ratio of the diastereomers formed can be used to obtain the enantiomeric ratio of the starting analyte mixture, provided that care is taken to avoid the pitfalls inherent in the indirect method [15].

Many drugs, intermediates in drug synthesis, and metabolites of drugs are primary amines, and thus sensitive enantiospecific analytical methods for compounds containing this functional group are of considerable importance. It is surprising, therefore, that the applicability of the enantiospecific OPA method to non-amino-acid primary amines in general and to drug analysis in particular, has been nearly completely ignored. Indeed, it appears in this regard that the enantiospecific OPA method has been nearly exclusively limited to amino acid analysis; the few exceptions to this generalization involve the application of the method to amino acid derivatives such as dipeptides and an amino acid ester [16] (poor resolution was obtained in the latter case), amino acid amides [17], and to amino alcohols mostly derived from α-amino acids [11,18]. It is indeed noteworthy in this regard that the only published attempt to resolve a chiral aliphatic amine failed [11].

Many primary amines of pharmacological interest are neither amino acids nor amino alcohols, and it is clear therefore that a broader applicability of the OPA/homochiral thiol method beyond the analysis of amino acids or their derivatives would be of considerable interest. Since the OPA derivatives are usually intensely fluorescent and are therefore detectable with high sensitivity, this method would be valuable in enantiospecific drug analysis in general, provided that the diastereomeric derivatives of drugs could be separated chromatographically. We therefore undertook to study the applicability of the enantiospecific OPA method to a variety of primary-amine drugs.

## EXPERIMENTAL

### *Chemicals*

o-Pthaldialdehyde, 1-thio-β-D-glucose (TG) sodium salt and N-acetyl-L-cysteine (NAC) were obtained from Sigma (St. Louis, MO, USA); N-acetyl-D-penicillamine (NAP) was obtained from Fluka (Buchs, Switzerland); (±)-*p*-chloroamphetamine, (±)-1-methyl-3-phenylpropylamine (3-amino-1-phenylbutane), 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (TATG), quinine sulfate, 9-anthraldehyde and ACS-grade sodium hydroxide were obtained from Aldrich (Milwaukee, WI, USA); (±)-tocainide was obtained from Astra Pharmaceutical (Worcester, MA, USA); (±)-, (+)- and (−)-mexiletine (MEX) were provided by Boehringer Ingelheim (Ingelheim, Germany); (±)-amphetamine, (−)-amphetamine and (±)-*p*-hydroxyamphetamine were obtained from Smith, Kline and French Labs. (Philadelphia, PA, USA); tranylcypromine was purchased from Regis (Chicago, IL, USA); rimantadine (RIM) was provided by Hoffmann-La Roche (Nutley, NJ, USA); HPLC-grade methanol and acetonitrile, and sodium borate decahydrate were from J. T. Baker (Phillipsburg, NJ, USA); ACS-grade glacial acetic acid was obtained from Fisher Scientific (Pittsburgh, PA, USA).

The synthesis of 3-amino-1-(4-hydroxyphenyl)-butane was described earlier by Gal *et al.* [19].

### *Drug, reagent and buffer solutions*

The derivatization solutions were prepared as follows: OPA (13.40 mg) and an equimolar amount of one of the four thiols were placed in a conical tube and dissolved in 1 ml methanol. The quantities of the thiols were: NAC: 16.3 mg; NAP: 19.1 mg; TG: 21.8 mg; TATG: 36.4 mg. The tubes were capped, protected from light and kept on ice. The solutions were kept refrigerated when not in use. Fresh solutions were prepared every other day.

The amine to be derivatized, in salt or free base form, was dissolved in 0.10 *M* hydrochloric acid solution to obtain a concentration of 0.25 mg of the amine as free base in 50 μl of solution.

A 0.1 *M* sodium borate buffer was prepared and the pH adjusted to 9.50 with a 2.0 *M* solution of sodium hydroxide. For the HPLC mobile phase, a sodium acetate buffer was prepared by diluting 3.0 ml glacial acetic acid to 1 liter with water and adjusting the pH of the buffer to 7.20 with a 2.0 *M* solution of sodium hydroxide. The buffers were vacuum filtered prior to use.

### *Derivatization procedure*

A 50-μl aliquot of the amine solution was placed in a conical centrifuge tube and was treated successively with 50 μl of the borate buffer and 100 μl of

the derivatization solution. The contents of the tube were swirl-mixed for one minute and the tubes were then placed on ice and protected from light. After a derivatization period of exactly 5 min the contents of the tube were diluted with 2 ml of mobile phase and aliquots of 5 μl were injected into the LC system. The tubes were continued to be kept on ice and protected from light.

*Determination of enantiomeric purity*

The enantiomeric purity of (+)-norephedrine was determined using the homochiral isothiocyanate 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) of high enantiomeric purity (> 99.90%) [20]. (+)-Norephedrine free base (1 mg) was placed in a conical tube. To the tube was added 3 mg TAGIT in 100 μl acetonitrile. The contents of the tube were swirl-mixed and derivatization was allowed to take place at room temperature for 30 min. The reaction mixture was diluted with 900 μl mobile phase (methanol–water, 47:53, v/v) and aliquots of 10 μl were injected into the LC system. The derivatives were monitored by UV detection at 254 nm.

(+)-Norephedrine was derivatized with OPA/NAC and analyzed as described above. The mobile phase employed consisted of methanol–HPLC buffer–acetonitrile mixed in the ratio of 40:60:1 (v/v/v), and the flow-rate was 1 ml/min. The eluent was monitored at 254 nm with UV detection.

*Studies on the formation and stability of MEX/NAC*

(±)-MEX HCl (1 mg) in 400 μl methanol was placed in a Reacti-Vial, and was treated successively with 400 μl borate buffer and 200 μl of the NAC derivatization solution. The derivatization was carried out as described above, and after a derivatization period of exactly 5 min, the external standard solution, containing 5 mg quinine sulfate in 200 μl of a 1:1 (v/v) solution of acetonitrile and ethanol, was added to the vial, the contents of the vial swirl-mixed and placed on ice in the dark. Aliquots of 100 μl were withdrawn from the Reacti-Vial at 5, 10, 15, 30, 60, 180 and 360 min, and immediately diluted with 2 ml of mobile phase. Aliquots of 10 μl of the diluted samples were injected into the LC system.

*Studies on the formation and stability of MEX/TATG*

(±)-MEX hydrochloride (1 mg) in 400 μl methanol was placed in a Reacti-Vial and derivatized with OPA/TATG as described in the preceding section. After the 5-min reaction time 100 μl of the external standard solution containing 9-anthraldehyde at a concentration of 1 mg in 10 ml acetonitrile was added to the vial, the contents of the vial swirl-mixed and placed on ice in the dark. Aliquots of 100 μl were withdrawn from the Reacti-Vial at 5, 10, 15, 30, 60, 180 and 360 min, and immediately diluted with 2 ml mobile phase. Aliquots of 50 μl were injected into the LC system.

*Chromatography*

The HPLC system consisted of a Waters Associates (Milford, MA, USA) Model U6K injector, a Model 6000A solvent delivery system and a Model 420 fluorescence detector equipped with a 338-nm excitation filter (band pass ± 6 nm) and a 425-nm long-pass emission filter. In some cases the fluorescence detector used was a Hitachi–Perkin-Elmer Model 204 A. The determination of the excitation and emission maxima of the RIM/TATG derivatives was carried out on a Waters Model 470 fluorescence detector. UV monitoring of the HPLC effluent was performed at 254 nm with a Waters Model 440 or a model Lambda-Max Model 480 detector.

The separations were carried out on a Waters Nova-Pak $C_{18}$ column, 150 mm x 3.9 mm, with 4 μm particle size. The mobile phases used for the separation of the amines (Table I) were prepared by

TABLE I

COMPOSITION OF MOBILE PHASES EMPLOYED FOR THE RESOLUTION OF PRIMARY-AMINE DRUGS

| MP[a] | Ratio[b] | MP[a] | Ratio[b] | MP[a] | Ratio[b] |
|---|---|---|---|---|---|
| A | 85:15:0 | G | 60:40:1.5 | M | 50:50:2.5 |
| B | 75:25:0 | H | 60:40:0 | N | 50:50:2.0 |
| C | 70:30:0 | I | 55:45:2.5 | O | 50:50:0.5 |
| D | 67:33:0 | J | 55:45:2.0 | P | 40:60:5.0 |
| E | 66:34:0 | K | 53:47:2.5 | Q | 40:60:2.0 |
| F | 65:35:0 | L | 50:50:5.0 | R | 30:50:20 |

[a] Mobile phase code for Table II.

[b] The ratio methanol–sodium acetate buffer, pH 7.20–acetonitrile, mixed v/v/v.

first vacuum filtering the individual components and then mixing them in the appropriate ratio. The buffer used was 50 m*M* sodium acetate, pH 7.20. The mobile phases were delivered at 1.0 ml/min, and the effluent was monitored with a fluorescence or a UV detector.

## RESULTS AND DISCUSSION

Nine primary amines of pharmacological interest were studied (first name given is that from Chemical Abstracts Service): α-methylbenzeneethanamine (amphetamine, AMP), 4-chloro-α-methylbenzeneethanamine (*p*-chloroamphetamine, PCA), 4-(2-aminopropyl)phenol (hydroxyamphetamine, HAM), α-methylbenzenepropanamine (3-amino-1-phenylbutane, APB), 4-(3-aminobutyl)phenol [3-amino-1-(4-hydroxyphenyl)butane, AHB], *trans*-2-phenylcyclopropanamine (tranylcypromine, TCP), 2-amino-N-(2,6-dimethylphenyl)propanamide (tocainide, TOC), 1-(2,6-dimethylphenoxy)-2-propanamine (mexiletine, MEX) and α-methyltricyclo [3.3.1.1$^{3,7}$]-decane-1-methanamine (rimantadine, RIM) (Fig. 1).

RIM is a simple aliphatic amine with a bulky alkyl group near the primary amine moiety. AMP, TCP and APB are simple arylalkylamines without any heteroatoms in their structure; HAM and AHB are the *p*-hydroxy derivatives corresponding to AMP and APB, respectively; it is noteworthy that

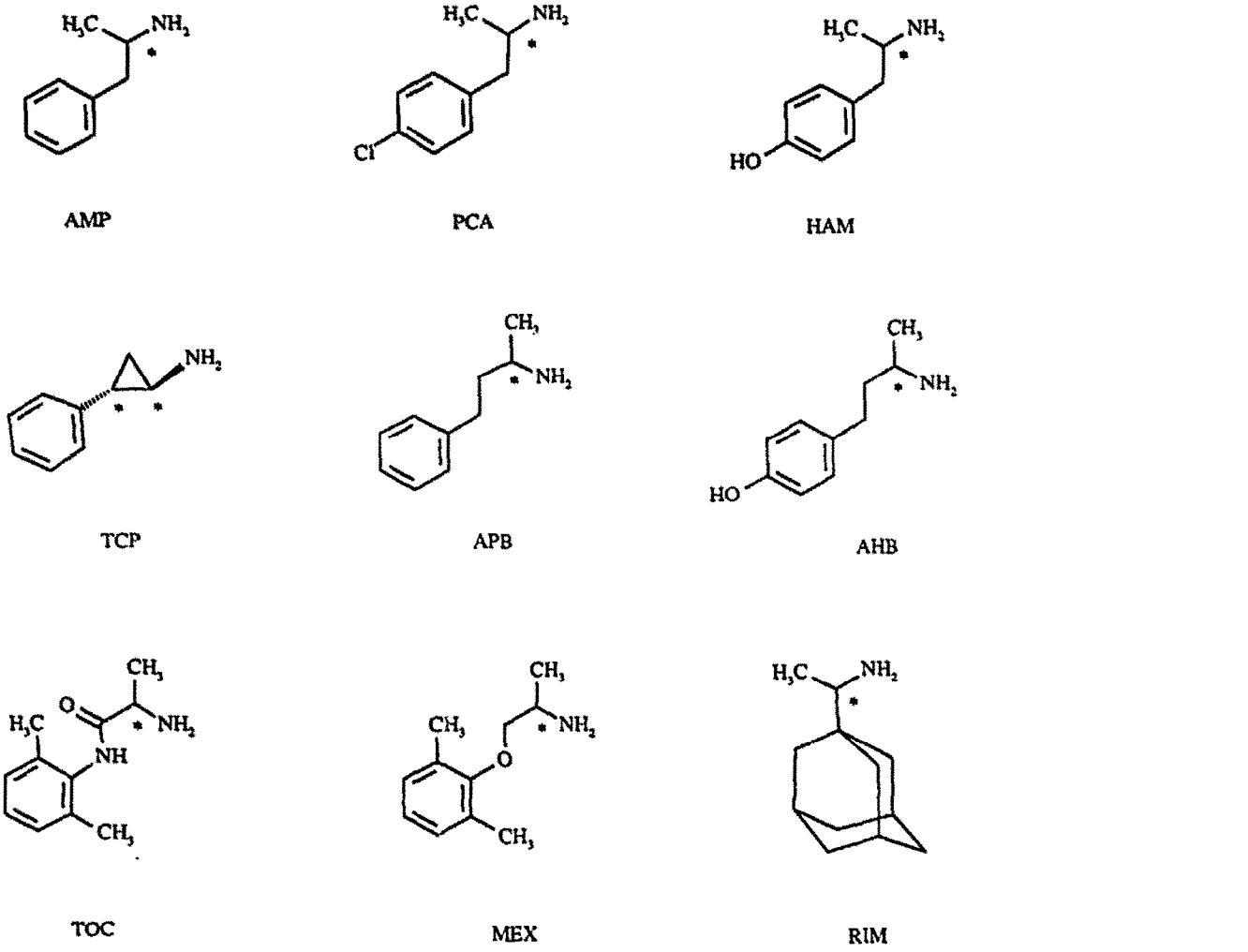


Fig. 1. The chemical structures of the drugs used in the derivatizations; the asterisk indicates the stereogenic center(s). The stereochemical bonds in the structure of TCP denote only relative (*trans*) configuration.

the hydroxyl group in these phenols is in a position remote from the primary amino group, unlike the hydroxyl group in the β-amino alcohols previously studied [11,18]. In PCA a *p*-chloro group is added to the basic structure of AMP. MEX contains an ether linkage between the aromatic ring and the side chain with the primary amino group, while TOC contains a carboxamide moiety between the aromatic ring and the primary amino functionality in the side chain. In all of the drugs the primary amino group is at the stereogenic center (in TCP, at one of the two stereogenic centers), and all but one (TCP) of the drugs have a methyl group at the stereogenic center which also bears the amino group; this is representative of many chiral drugs, inasmuch as a review of the *USAN and USP Dictionary of Drug Names* [45] indicates that the vast majority of chiral primary amines that are not amino acids or derivatives thereof have such an arrangement at the stereogenic center.

From the pharmacological standpoint, the drugs selected represent a variety of classes. AMP, PCA and HAM are based on the 1-phenyl-2-aminopropane (amphetamine) structure; such drugs display a variety of pharmacological activities [21–23] depending on structural details, and stereochemistry plays an important role in the actions and biological disposition of such drugs [24–26]. HAM is not only a drug in its own right but is also a metabolite of AMP in some species [27]. APB is a sympathomimetic amine, and both APB and AHB are metabolites of the antihypertensive drug labetalol [19]. Furthermore, we have found that the metabolism of labetalol to APB is stereoselective [28]. It is also noteworthy that the basic skeleton of APB and AHB —the 3-amino-1-phenylbutane moiety— is present in a number of drugs in addition to labetalol, *e.g.*, bufeniode, butopamine, dobutamine, medroxalol, sulfinalol, etc. In analogy with labetalol, some of these drugs may be metabolized by N-dealkylation, a common biotransformation, to the primary amine APB, AHB, or a derivative thereof, and thus the chromatographic resolution of the enantiomers of these compounds is of interest. TCP is an inhibitor of the enzyme monoamine oxidase (MAO), and is used as an antidepressant drug. The two enantiomers of TCP differ in their biological effects [29]. TOC and MEX are cardiac antiarrhythmic drugs that display enantiomeric differences in their actions [30] and disposition [31–33]. RIM is an antiviral agent used clinically as the racemate; preliminary evidence was recently published suggesting that the drug may undergo stereoselective metabolic conjugation [34].

Four chiral thiols were used in the derivatizations: N-acetyl-L-cysteine (NAC), N-acetyl-D-penicillamine (NAP), 2,3,4,6,-tetra-O-acetyl-1-thio-β-D-glucopyranoside (TATG), and 1-thio-β-D-glucose (TG) (Fig. 2). NAC and NAP are α-amino-acid derivatives, while TATG and TG are derivatives of glucose. All four reagents are commercially available in the homochiral form.

In general, it is often observed that homochiral reagents that are natural products or derivatives thereof, are available in high enantiomeric purity. It is noteworthy in this regard that all four reagents used in the present work are based on natural products. The enantiomeric purity of a sample of TATG obtained from Sigma has been previously determined [12] using homochiral tyrosine to be 99.9%. Our sample of TATG was also from Sigma, and we did not examine in detail its enantiomeric purity; however, derivatization of homochiral samples of AMP and MEX with TATG followed by chromatographic analysis of the derivatives showed that the reagent was at least 99.0% enantiomerically pure, as 1.0% of enantiomeric contamination would have been detectable under our chromatographic conditions.

The enantiomeric purity of NAP obtained from Fluka has been previously determined using homochiral amino acids and has been found to be 99.9% [11]. Our sample of NAP was also from Fluka, and analysis of AMP and TOC with the reagent showed that it was at least 99.0% enantiomerically pure.

In order to determine the enantiomeric purity of NAC, the reagent was reacted with OPA and (+)-norephedrine of known enantiomeric purity, and the product mixture was analyzed by chromatography. The enantiomeric purity of the norephedrine sample was determined via derivatization of norephedrine with the homochiral isothiocyanate TAGIT [20]; this reagent is based on D-glucose and is enantiomerically pure within experimental determination. Using this method the enantiomeric purity of NAC was found to be >99.0%.

The enantiomeric purity of TG used previously in the HPLC resolution of amino acids [35] or amino

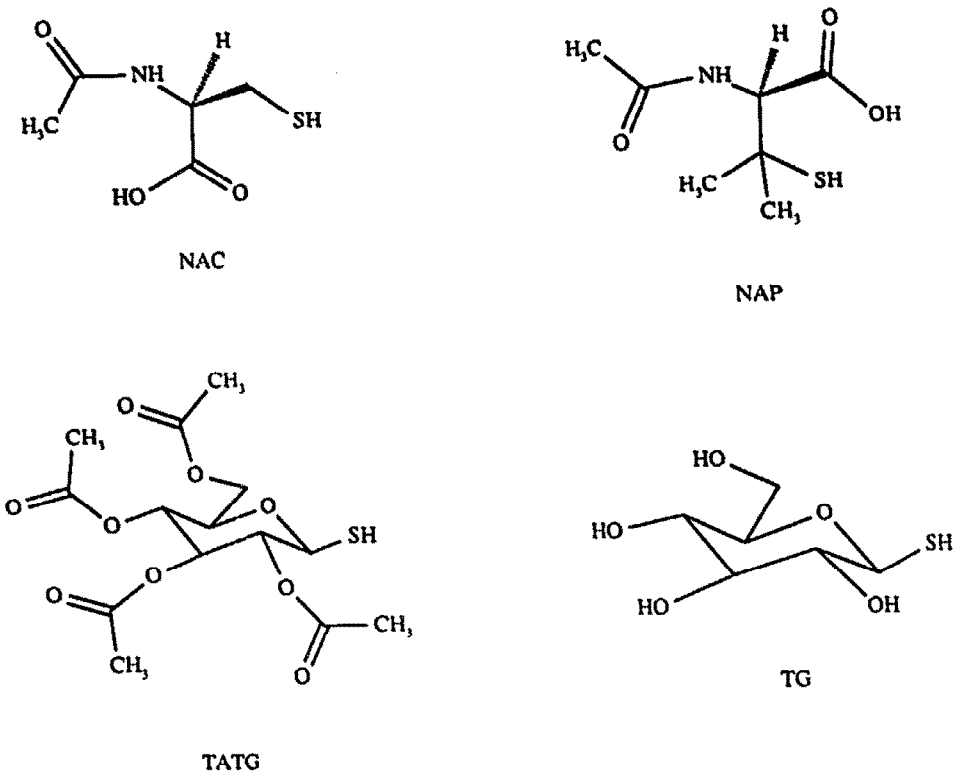


Fig. 2. The chemical structures of the homochiral thiols used as derivatizing agents.

alcohols [18] was not specified. We also did not examine the enantiomeric purity of TG, but it was clear from the analysis of the single-enantiomeric forms of AMP that the reagent was at least 99.0%.

The reaction of a racemic primary amine with OPA and an optically active thiol produces two diastereoisomeric isoindoles; this reaction is known to be rapid, and is complete in a few minutes or less [11,14]. The chemistry of the reaction of OPA and NAP with (±)-AHB is shown in Fig. 3. We examined the time course of the reaction of NAC and of TATG with (±)-MEX. After 5 min, the first time point, there was no further product formation; the derivatives were found to be stable over a period of 6 h while kept in ice in the dark (Fig. 4).

A great deal of data in the literature shows that the OPA derivatives of amino acids and related primary amines with thiols are generally highly fluorescent, and excitation and emission wavelengths in the ranges of *ca.* 330–350 and 400–480 nm, respectively, are typically monitored during the analysis of OPA derivatives, the exact values in each case depending on the identity of the thiol, the amine, and the solution medium. We determined the excitation and emission maxima of the TATG derivatives of RIM to be 338 nm and 420 nm, respectively, values very similar to 342 and 410 nm, respectively, found for the TATG derivatives of amino acids [12].

The resolution of the nine drugs with the four reagents is summarized in Table II. The separations were achieved on Waters Nova-Pak octadecylsilane HPLC columns, and other brands were not evaluated. The thiols varied in their ability to resolve the nine drugs (see below), but it was found that for all but one amine (TCP) at least one homochiral reagent provided suitable resolution, *i.e.*, baseline separation (resolution factor $R_s \geq 1.50$ [37]) within a reasonably short run time (Table II). For TCP, a near-baseline value of 1.30 was obtained for the resolution factor $R_s$ with NAC at retention times of *ca.* 25 min. Representative examples of separations are shown in Fig. 5.

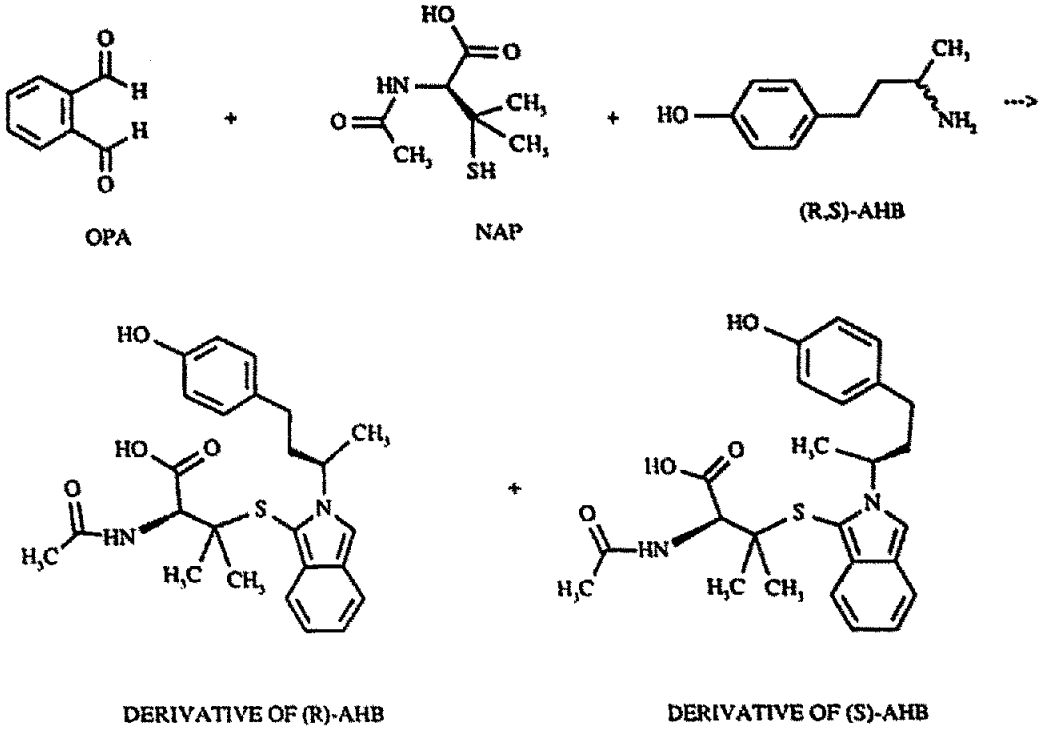


Fig. 3. The reaction of racemic AHB with OPA and NAP to produce the two diastereomeric isoindole derivatives.

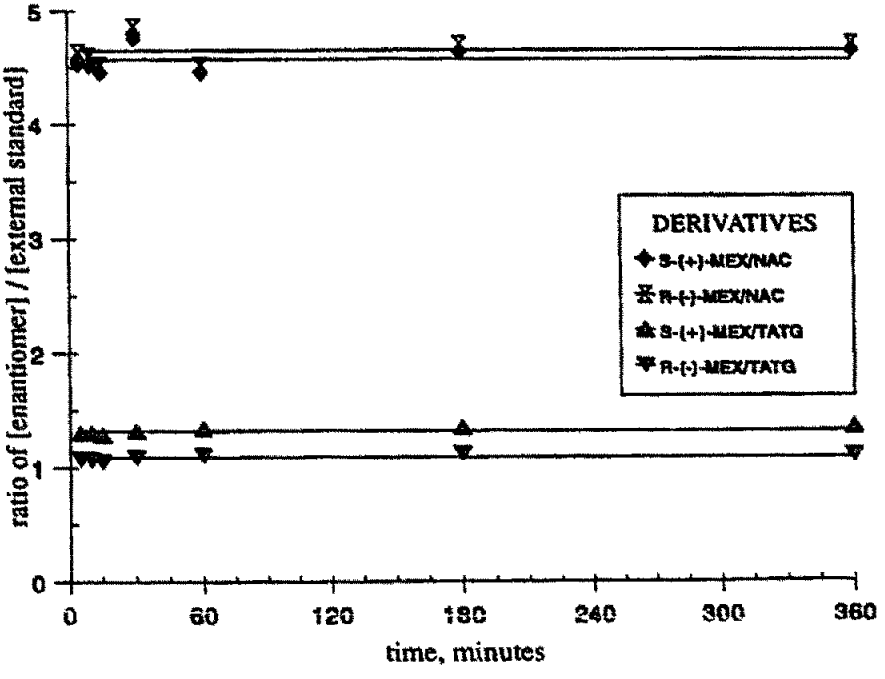


Fig. 4. The formation and stability of the MEX NAC and the MEX TATG derivatives; each point is the mean of duplicate determinations.

TABLE II

SEPARATION OF ENANTIOMERIC PRIMARY-AMINE DRUGS AS THEIR ISOINDOLE DERIVATIVES ON A WATERS $C_{18}$ COLUMN

| Drug | Thiols | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NAC | | | NAP | | | TATG | | | TG | | |
| | $\alpha^a$ | $R_s^b$ | $t_R^c$ | $\alpha^a$ | $R_s^b$ | $t_R^c$ | $\alpha^a$ | $R_s^b$ | $t_R^c$ | $\alpha^a$ | $R_s^b$ | $t_R^c$ |
| AMP | 1.17[M] | 2.58 | 16.98 *R*-(−) | 1.12[I] | 1.61 | 15.41 *S*-(+) | 1.35[B] | 4.33 | 6.48 *R*-(−) | 1.15[I] | 2.19 | 12.37 *R*-(−) |
| HAM | 1.22[M] | 2.67 | 6.11 *S*-(+) | 1.10[M] | 1.31 | 11.24 *R*-(−) | 1.07[G] | 1.17 | 25.66 *R*-(−) | 1.14[N] | 1.80 | 8.84 *S*-(+) |
| PCA | 1.15[G] | 2.09 | 8.57 | 1.15[J] | 1.97 | 22.83 | 1.47[D] | 6.10 | 7.95 | 1.14[G] | 2.10 | 12.31 |
| APB | NR[d,H,R] | NR | 8.00 | 1.07[R] | 1.50 | 24.43 | NR[H] | NR | 12.00 NR | NR[J] | NR | 28.70 |
| AHB | 1.25[I] | 2.56 | 4.63 | 1.11[K] | 1.64 | 10.94 | 1.07[G] | 1.32 | 36.27 | 1.17[N] | 2.32 | 12.69 |
| MEX | 1.11[G] | 1.71 | 12.38 *S*-(+) | 1.08[J] | 1.08 | 25.73 *R*-(−) | 1.19[B] | 3.00 | 14.69 *S*-(+) | NR[G] | NR | 22.94 |
| TOC | 1.11[Q] | 1.78 | 20.32 *S*-(+) | 1.13[O] | 2.00 | 12.75 *R*-(−) | NR[C] | NR | 5.84(NR) | 1.10[P] | 1.64 | 22.12 *S*-(+) |
| TCP | 1.13[N] | 1.30 | 25.81 | 1.07[L] | 1.10 | 30.11 | 1.07[D] | 1.31 | 30.02 | 1.06[J] | 1.02 | 25.42 |
| RIM | 1.03[F] | 0.34 | 23.87 | 1.10[L] | 1.35 | 17.78 | 1.24[A] | 2.61 | 5.88 | NR[C] | NR | 15.01 |

[a] Separation factor, see ref. 36; capital letter in superscript indicates mobile phase composition given in Table I.
[b] Peak resolution, see ref. 37.
[c] Retention time of first eluting peak; if known, configuration of corresponding enantiomer is indicated; if no resolution, retention time of single peak.
[d] NR = No resolution.

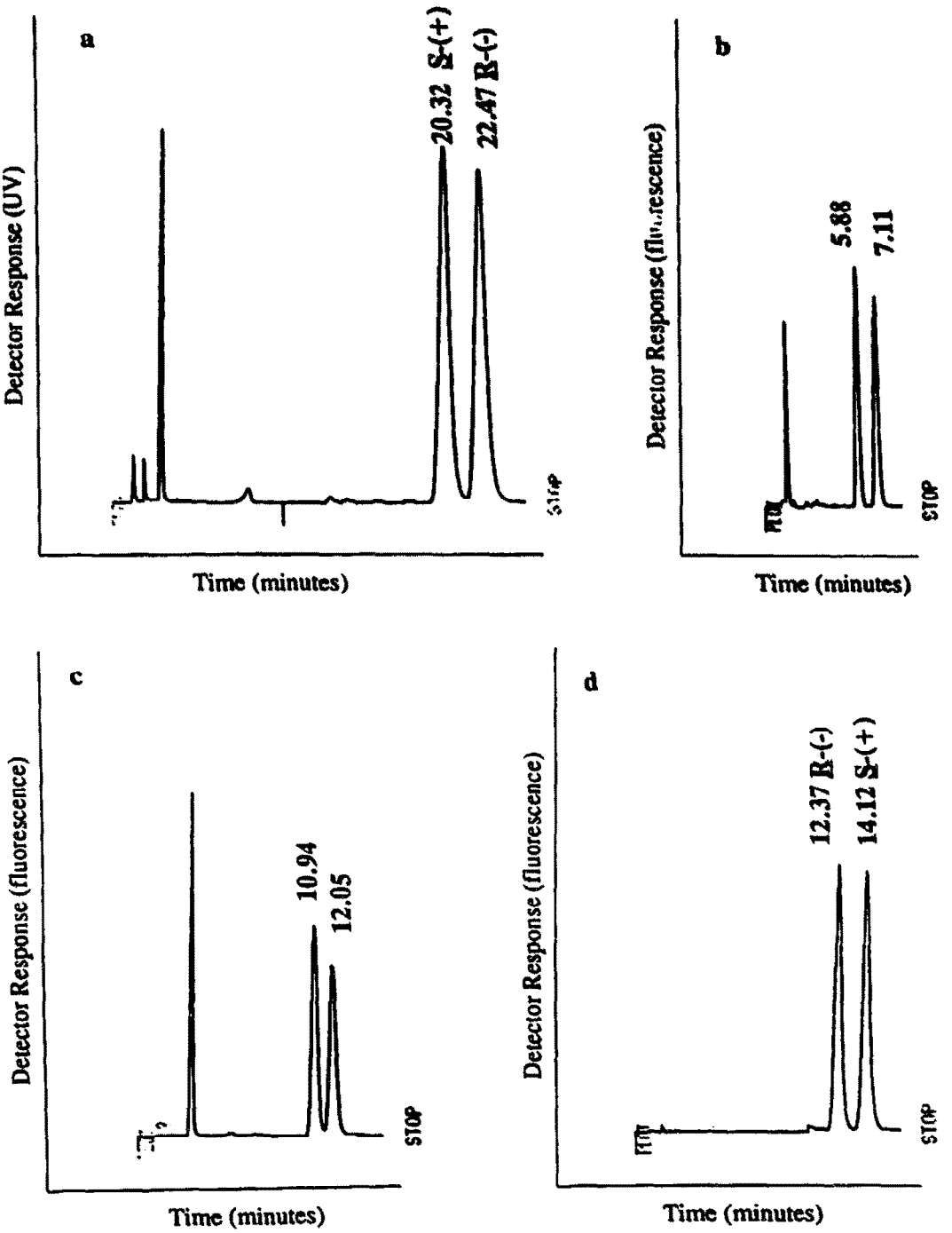


Fig. 5. The resolutions of (a) TOC with NAC; (b) RIM with TATG; (c) AHB with NAP; (d) AMP with TG. Retention times in minutes are shown, and the order of elution of the derivatives is also given when known. For chromatographic conditions see Experimental and Table 1.

In general, good peak shapes were obtained using mixtures of a sodium acetate buffer pH 7.2, methanol, and acetonitrile. The composition of mobile phase is important, as we found that other mobile phases, *e.g.* a 0.02 *M* ammonium phosphate buffer, pH 4.50, gave broad and poor chromatographic

TABLE III

DIASTEREOMERIC ISOINDOLE DERIVATIVES WITH UNEQUAL FLUORESCENCE AND/OR UV DETECTOR RESPONSES

| Drug/thiol | % Relative size[a,b] of smaller peak | |
|---|---|---|
| | Fluorescence | UV |
| AMP/NAC | [c] | 95.14 ± 0.30 |
| AMP/NAP | 80.25 ± 0.45 | [c] |
| AMP/TATG | 88.27 ± 1.36 | 97.37 ± 0.91 |
| AMP/TG | 92.48 ± 0.63 | [c] |
| HAM/NAC | [c] | 95.67 ± 1.01 |
| HAM/TG | 95.77 ± 0.33 | 95.13 ± 2.02 |
| PCA/NAC | [c] | 90.37 ± 0.32 |
| PCA/NAP | [d] | 97.02 ± 0.31 |
| PCA/TATG | 90.59 ± 0.92 | [c] |
| PCA/TG | 93.07 ± 0.18 | [c] |
| APB/NAP | 92.07 ± 0.67 | [d] |
| AHB/NAC | 95.00 ± 0.42 | 95.24 ± 0.50 |
| AHB/NAP | 91.98 ± 0.37 | 97.80 ± 0.50 |
| AHB/TG | 97.40 ± 0.53 | [c] |
| MEX/NAC | [c] | 94.27 ± 0.58 |
| MEX/TATG | 81.58 ± 1.22 | 93.49 ± 1.09 |
| TOC/NAC | 62.67 ± 1.26 | 91.91 ± 0.23 |
| TOC/NAP | 80.83 ± 0.65 | 95.37 ± 0.65 |
| TOC/TG | 68.46 ± 0.30 | 81.75 ± 1.30 |
| RIM/TATG | [c] | 70.71 ± 0.96 |

[a] Defined by setting larger diastereomeric peak area = 100% and calculating relative size of the other diastereomeric peak.
[b] Mean ± S.D., $n \geq 3$. No determination of the relative peak sizes could be made if the peaks were not completely resolved.
[c] Value ≥ 98%, considered not significantly different from 100% peak.
[d] Not evaluated.

peak shapes. Furthermore, it has also been reported that the fluorescence intensity and stability of some OPA derivatives are dependent on the nature and pH of the medium [38]. We observed that the presence of acetonitrile in the mobile phase improved the peak shapes, but in a few cases (Table I) acetonitrile was found to be unnecessary because good peak shapes were obtained using only mixtures of the buffer and methanol.

Individual enantiomers were available for AMP, HAM, TOC, and MEX, and were used to determine the order of elution of the diastereomeric derivatives (Table II). All four of these drugs have the *S*-(+)/*R*-(−) absolute configuration. The NAC-derivative of the (+)-enantiomer of HAM, MEX and TOC eluted before the derivative of the corresponding (−)-enantiomer, while the reverse order was seen for AMP (Table II). For all four amines the order of elution of the derivatives was reversed when NAP was the derivatizing agent, a reversal that is not surprising since NAP and NAC have opposite configurations, NAC being the L enantiomer while NAP is the D isomer. The levorotatory enantiomer of AMP eluted first after derivatization with TATG or with TG. Interestingly, however, there was a reversal of the order of elution of the HAM derivatives between TATG and TG. Our data obtained with the individual enantiomers also indicated that no racemization of the starting materials (*i.e.* the thiol and the drug) or epimerization of the derivatives occurred, since a single peak was obtained in every case where a single enantiomer was derivatized. Detailed studies of the mechanisms of the chromatographic diastereoselectivity will have to be carried out before we can predict the order of elution of the diastereomers.

It was observed in several cases that the two diastereomeric derivatives exhibited unequal fluorescence intensities (Table III). Among the potential causes of this phenomenon are unequal rates of formation of the two derivatives (kinetic resolution), unequal rates of decomposition of the formed derivatives, and an unequal response by the detector employed. The derivatizing reagents are used in excess with respect to the amine, and the reaction is known to be rapid, usually complete in a few minutes [11,14]. It is clear, therefore, that unequal formation rates could not account for the unequal fluorescence intensities. This conclusion is confirmed by the data presented in Fig. 4. Furthermore, Fig. 4 also indicates that diastereoselective decomposition of the derivatives also does not occur and thus cannot explain the differences observed. These considerations and results suggest therefore that unequal peak areas were the result of unequal response by the fluorescence detector, *i.e.*, the diastereomers differ in their fluorescence properties. This conclusion is further supported by similar observations reported for some amino acid OPA derivatives [9,11,13]. When UV absorption at 254 nm was used to monitor the column effluent, it was found that the difference between the diastereomeric responses were considerably smaller in most cases, and for several amines reverted to the 1:1 ratio (Table III). Interestingly, however, in several cases fluorescence detec-

tion gave equal-area peaks for the diastereomers, while UV detection gave unequal peak areas (Table III). It is also to be noted that for all of the drugs except TOC, APB and AHB, at least one of the derivatizing agents produced diastereomers that exhibited equal fluorescence.

Unequal detector response to diastereomeric derivatives, when it occurs, is a significant disadvantage of the indirect approach to chiral separations. It is indeed undeniable that an equal response to the derivatives is highly preferable, and this is one of the factors that make the *direct* approach to enantiospecific analysis (*i.e.* homochiral stationary or mobile phase) superior in principle to the indirect method. It should be noted, however, that an unequal detector response does not necessarily render an indirect separation useless in many applications, because in quantitative enantiospecific drugs analysis it is usually necessary to base the quantification individual calibration curves for the enantiomers, a procedure that accounts for unequal detector response. The occurrence of unequal detector response by a given pair of diastereomeric derivatives can be determined without difficulty by derivatizing the racemate and ruling out other potential sources of unequal peak heights, as we did in the present study (see above).

It was observed qualitatively that the NAC derivatives appeared considerably more fluorescent than those of the other reagents. This phenomenon was not examined quantitatively, but similar observations have also been reported for amino acids. Euerby *et al.* [39], for example, found that the NAC derivatives of some amino acids were more intensely fluorescent than the corresponding NAP derivatives. Such differences may be important in maximizing the sensitivity of the method, and therefore deserve further study.

The minimum detectable amount was 120 pg for each enantiomer of MEX/NAC derivative at a signal-to-noise ratio of 3:1 when the Waters Model 420 detector was used. With a more sensitive fluorescence detector equipped with a xenon lamp (Hitachi–Perkin-Elmer) the minimum amount detectable was 75 pg per enantiomer. The minimum amount of RIM/TATG derivatives detectable using UV absorption at 254 nm was 6 ng per enantiomer with a signal-to-noise ratio of 3:1.

The purpose of our study was to explore the applicability of the enantiospecific OPA method to non-amino-acid drugs and to establish suitable conditions for such analyses, and a systematic investigation of the structure–resolution relationships of the method remains to be carried out. Nevertheless, our data allow several observations about the influence of structural elements on the resolution. In general, the amphetamine derivatives (AMP, PCA, HAM) were well resolved with all of the reagents, with the exception of HAM, which was not well resolved with TATG and only slightly better with NAP (Table II). In HAM the *para*-substituent on the phenyl ring is electron-donating (OH) and in PCA it is electron-withdrawing (Cl) with respect to the unsubstituted analogue AMP, but a uniform relationship between diastereoselectivity ($\alpha$ in Table II) and the electronic properties of the substituents was not discernible. AMP differs structurally from APB in that in the latter an additional methylene group separates the chiral center (and the amino group) from the phenyl ring (Fig. 1). As seen in Table II, in every case better selectivity ($\alpha$) was obtained for AMP than for APB. Indeed, APB was poorly or not at all resolved with three of the four reagents, while good to excellent resolution was achieved for AMP with all four reagents. These observations indicate an important role for the phenyl ring of the analyte in the separation, and show that its distance from the chiral center or from the amino group is important for the separation of the diastereomers. It has been suggested [40] that diastereomer separations depend on intramolecular interactions (*e.g.*, via hydrogen bonding, hydrophobic forces, dipole interactions, etc) that result in a reduction of the conformational mobility of the molecule, which may in turn result in increased physicochemical differences between the diastereomers. Such differences are, of course, responsible for the chromatographic separation. Clearly, the ability of the phenyl ring to participate in intramolecular interactions (*e.g.* via its $\pi$-electron system) is dependent on its particular position in the molecule, and our data show that the phenyl ring is better disposed in AMP than in APB to enhance the separation.

That the situation is rather complex, however, is shown by an analogous comparison of the diastereomeric separations of the derivatives of HAM and AHB, *i.e.*, the *p*-hydroxy analogues of AMP

and APB, respectively. As seen in Table II, in one case (TATG derivatives) the α values for the two drugs were identical, and for each of the remaining three reagents the α value of the AHB derivatives was always greater than that of the HAM derivatives. Thus, in a reversal of the findings for the AMP/APB pair, the *p*-hydroxyphenyl ring in AHB is in general better able to influence the separation —possibly through some diastereoselective intramolecular interaction— than the *p*-hydroxyphenyl ring of HAM. Also relevant in this regard is the observation (Table II) that the derivatives of AHB were always better separated (as judged by the α values) than those of APB, suggesting that the introduction of the *p*-hydroxy group either diastereoselectively enhanced the particular intramolecular interaction involved, or in fact caused the predominance of an entirely different interaction. When the AMP/HAM pair is similarly examined (Table II), however, it is seen that the introduction of the *p*-hydroxy group into the amphetamine structure caused a *decrease* in the α value in three of the four cases, suggesting that the potential intramolecular interaction(s) involved may be significantly different from those operating in the APB/AHB case. Furthermore, introduction of the *p*-hydroxy group into the amphetamine structure also caused the reversal of the order of elution of the derivatives for three of the four homochiral derivatizing agents (Table II), suggesting a change in the nature of the predominant interaction(s).

It is also instructive to compare the resolutions of TCP with those of AMP. The former compound, *trans*-phenylcyclopropylamine (Fig. 1), may be considered a rigid analogue of AMP. An inspection of the relevant α values (Table II) shows that in all cases AMP was better resolved than TCP, suggesting that the conformational requirements for the resolution of AMP are not well mimicked by the rigid analogue TCP. Clearly, further detailed studies will have to be carried out to explain fully the role of physicochemical and structural factors in the above separations.

It is also interesting to consider the role of the chemical structure of the homochiral thiols in the resolutions. The two amino acid derivatives NAC and NAP are closely related, the latter being the β,β-dimethyl analogue of the former (Fig. 2). The two sugar derivatives are also related, TATG being the tetraacetylated analogue of TG (Fig. 2). TATG is clearly less polar and more hydrophobic than TG. To obtain a rough index of resolving ability for the reagents, we calculated the mean α value for each thiol, using α = 1.00 when no resolution was obtained. The results were: TG mean α = 1.08, NAP 1.10, NAC 1.13, TATG 1.16. Thus, by this standard, the best reagent was TATG, *i.e.* a molecule with a bulky hydrophobic group attached to the thiol moiety, in agreement with suggestions that such groups can maximize physicochemical differences between the diastereoisomers [40]. It is interesting in this context that TG, probably the most hydrophilic molecule among our reagents, proved to be the poorest reagent in our separations, raising the question of the role of lipophilicity of the reagent in the resolutions. However, that lipophilicity may not be an overriding factor is indicated by the comparison between NAP and NAC: the former compound, being a dimethyl derivative of the latter, is more hydrophobic than the latter, but exhibits a lower mean α value. Our limited data do not, however, allow detailed conclusions about the role of the structure of the thiol in the resolutions. It should be emphasized also that the mean α value for a reagent is only an average index of resolving power that does not predict the separation achieved for any specific racemate. Consider, for example, the case of APB: this compound could only be resolved with NAP. It is noteworthy in this context that several other homochiral thiols have been employed in separations of enantiomeric amino acids, *e.g.*, a series of N-acylcysteines [41]. While some of these reagents produced good resolutions of some amino acid enantiomers, they suffer from the major disadvantage that they are not commercially available.

As mentioned earlier, a previous attempt [11] to resolve a chiral aliphatic amine failed, prompting the authors to speculate that the hydroxyl or carboxyl group of an amino alcohol or amino acid, respectively, is required to separate the diastereomeric OPA derivatives of chiral amines. Such suggestions are incompatible with our results, as our data clearly show that the OPA/homochiral thiol method is applicable to a variety of primary amines, including alkyl- and arylalkylamines.

It is also clear from the above discussion that in applying the enantiospecific OPA method to a given compound the analyst should adopt a flexible and

empirical approach. Thus, a given racemate may be resolved with several thiols, but the resolution should be optimized with regard to extent of separation, detector response, analysis time, etc., by the judicious selection of the homochiral reagent.

Pre-column derivatization of primary amines with OPA-homochiral thiols offers several advantages: commercially available reagents and chromatographic columns are used; the reaction is simple and rapid, specific to primary amines, and is applicable to a variety of primary-amine drugs; the derivatives are generally highly fluorescent and the reagents are non-fluorescent, providing for sensitive and specific detection, and the procedure can be readily automated [11]. Furthermore, the OPA isoindole derivatives have also been shown to be electrochemically active [42,43], thus widening the scope of this approach. A significant disadvantage of the method is the possibility of unequal detector response. This was observed for some of the diastereomeric pairs in this study, and both fluorescence and UV detection suffered from such differences, the former more frequently and to a greater extent than the latter. It is important to emphasize, however, that for a majority of the amines at least one thiol provided diastereomers with equal detector response. Furthermore, even when unequal detector response is unavoidable, suitable calibration curves readily allow quantification of the enantiomers.

There are many therapeutic agents that are primary amines. Furthermore, many non-primary-amine drugs are metabolized to metabolites that are primary amines, via such biotransformations as N-dealkylation, amide hydrolysis, reduction of azo, nitro, nitroso and hydroxylamino groups, etc. It is reasonable to expect, therefore, that this method will be valuable in the area of enantiospecific pharmaceutical analysis and in drug metabolism and pharmacokinetic studies. It is interesting to note here that a preliminary attempt to apply the OPA, homochiral thiol to the enantiospecific analysis of a drug —baclofen, a γ-amino acid— was described [44] several years ago; while the procedure appeared promising, difficulties in the derivatization reaction were encountered that could not be explained [44]. Nevertheless, our results suggest that the OPA method should be further investigated in enantiospecific drug analysis.

In summary, then, the OPA, homochiral thiol method of enantiospecific analysis is applicable to a variety of primary-amine drugs representing a variety of pharmacological classes. We are currently investigating applications of the method to problems of stereoselective drug disposition.

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