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UNEXPECTED PHENOMENON IN THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF RACEMIC ^{14}C-LABELLED NICOTINE: SEPARATION OF ENANTIOMERS IN A TOTALLY ACHIRAL SYSTEM

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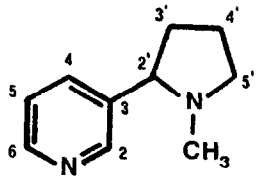
SUMMARY

Two different forms of racemic ^{14}C-labelled nicotine exhibit unusual behavior on Partisil-ODS and Partisil-SCX high-performance liquid chromatography systems, when co-injected with unlabelled nicotine of varying enantiomeric composition. Alone, either radiolabelled compound elutes as a single radioactive peak. In the presence of unlabelled (*S*)-(−)-nicotine or its antipode as standard, the ^{14}C activity is divided into two equal peaks. If unlabelled racemic nicotine is used as standard, only one ^{14}C peak is produced. Experiments were performed which demonstrate that the two ^{14}C peaks observed are due to the separated enantiomers of the radiolabelled material. The phenomenon responsible for this separation is not known, but may be due to differential enantiomeric association between like and unlike optical isomers of nicotine. The resulting effect provides a facile method for the isolation of the pure enantiomers of ^{14}C-labelled nicotine, on a totally achiral system. This method may also be applicable to other optically active radiolabelled compounds.

INTRODUCTION

The tobacco alkaloid nicotine (I) contains one asymmetric centre, at the 2′-C atom of the pyrrolidine ring. Naturally occuring nicotine exists entirely as the (*S*)-(−)-enantiomer and consequently this is the only commercially available form. As a result, the majority of metabolic studies performed to date have utilized only the (−)-isomer[1]. However, the results of those few studies which have involved (*R*)-(+)-nicotine suggest a marked stereospecificity in certain metabolic pathways, most



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notably N-oxidation[2,3]. In addition, much work has been accomplished in the study of receptor binding and pharmacological activity of nicotine[4–6] and the few studies involving (+)-nicotine indicate a significant pharmacological stereospecificity[7,8].

At present, (+)-nicotine is only available from the resolution of racemic nicotine. This may be prepared in two ways: (a) by total synthesis via a planar intermediate, such that both isomers are produced in equal quantities[9]; (b) by chemical racemisation of the naturally occuring (−)-isomer[10]. Resolution of the racemic material can then be achieved by selective fractional crystallization of one diastereoisomeric salt, formed by reaction with a chiral acid, *e.g.* di-(*p*-toluoyl)-(−)-tartaric acid[8]. The resulting crystalline product can be purified by recrystallization and then reconverted back to its free base form. However, this method is laborious and subject to high losses of material.

Both metabolic and receptor binding studies would benefit substantially from the availability of the pure (+)- and (−)-isomers of a radiolabelled form of nicotine. This would allow observation of very low levels of metabolites or receptor-nicotine complexes. The preparation of optically pure (+)- and (−)-enantiomers of 4′,4′-ditritio-nicotine has recently been achieved, and preliminary binding and distribution studies performed[11,12]. However, all direct syntheses of ^{14}C-labelled nicotine (which is often less metabolically labile than ^{3}H-labelled) reported to date have involved the formation of an intermediate which affords a racemic product[13,14]. Resolution of these products by chemical means is not possible, since with a radiolabelled molecule of high specific activity, the absolute quantity of material present is not normally sufficient for fractional crystallization.

It is feasible that a pure ^{14}C-labelled isomer of nicotine may be prepared from optically pure nornicotine. However, naturally occurring nornicotine is not optically pure, and its resolution can only be achieved via the synthesis of a precursor which is more susceptible to fractional crystallization[15]. Many studies appeared in the literature in the 1960's which claim to have used [^{14}C-NCH$_3$]-(−)-nicotine[16–21]. However, consideration of the source of this material[22] shows that it was obtained from methylation of natural nornicotine. Thus, all this work was presumably performed on a partially racemic labelled material.

It is therefore necessary to find a practical means of separating very small amounts of racemic radiolabelled nicotine. In recent years, various techniques have been developed which employ high-performance liquid chromatography (HPLC) for the separation of enantiomeric mixtures[23]. These methods may be divided as follows:

(1) Indirect separation: by the formation of diastereoisomeric salts or complexes, which can be separated on a conventional HPLC system.

(2) Direct separation: (a) using a chiral eluent in the mobile phase, which forms a different diastereoisomeric solvate with each enantiomer of the solute. These then have different affinities for a conventional HPLC support; (b) using a chiral support, such that a temporary diastereoisomeric association is formed between the enantiomers and the chiral moieties of the column.

All of these methods require that some component of the HPLC system, in addition to the enantiomers themselves, have some degree of chirality.

In our laboratory, we have undertaken the study of the *in vivo* metabolism of ^{14}C-labelled nicotine, by HPLC analysis of metabolites found in the urine[24]. Two different forms of ^{14}C-labelled material were used in the study ([^{14}C-NCH$_3$]-(±)-

nicotine-(+)-bitartrate and [^{14}C-2']-(±)-nicotine free base), and despite their different sources, both were racemic mixtures. When these radiolabelled compounds were analyzed for radiochemical purity by HPLC, in the presence of cold (unlabelled) (−)-nicotine standard, both samples exhibited an unexpected splitting of the radioactivity into two distinct peaks. In the absence of cold nicotine standard, either form of radiolabelled material gave only one radioactive peak. This phenomenon could be demonstrated on two entirely different HPLC systems. This observation led us to examine the behavior of the above radiolabelled samples in the presence of varying proportions of the two unlabelled enantiomers of nicotine. The results of this study suggest that it is possible to resolve racemic ^{14}C-labelled nicotine into its enantiomers using an entirely achiral HPLC system, by coinjection with one pure enantiomer of cold nicotine.

MATERIALS AND METHODS

Materials

[^{14}C-2']-(±)-Nicotine (0.25 mCi, specific activity 60 mCi/mmole), was obtained from New England Nuclear (Boston, MA, U.S.A.); [^{14}C-NCH$_3$]-(±)-nicotine-(+)-bitartrate (0.25 mCi, specific activity 57 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, U.K. Stock solutions in methanol (1 μCi/ml) were prepared for both labelled compounds.

S-(−)-Nicotine, di-(*p*-toluoyl)-(−)-tartaric acid, di-(*p*-toluoyl)-(+)-tartaric acid and sodium acetate (gold label) were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC grade methanol was supplied by Fisher (Fair Lawn, NJ, U.S.A.). Ammonium hydroxide and glacial acetic acid used in the preparation of buffers were of reagent grade, supplied by Fisher. Two forms of scintillation cocktail were used; 3a70B was obtained from RPI (Mount Prospect, IL, U.S.A.), and Flo Scint III was obtained from Radiomatic (Tampa, FL, U.S.A.).

[^{3}H-NCH$_3$]-(+)-nicotine (1 mCi, specific activity 76.5 Ci/mmole) and [^{3}H-NCH$_3$]-(−)-nicotine (1 mCi, specific activity 68.6 Ci/mmole) were gifts from New England Nuclear (Boston, MA, U.S.A.). These compounds are not yet commercially available.

Racemic nicotine was a gift from Dr. T. S. Osdene, Philip Morris, U.S.A., Research Center (Richmond, VA, U.S.A.). In addition, racemic nicotine was synthesized from (−)-nicotine by the method of Bowman *et al.*[10] and by total synthesis via nornicotine[24]. (*R*)-(+)-Nicotine-D-ditartrate was a gift from Drs. Edward R. Bowman and Everette L. May, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA, U.S.A. (*R*)-(+)-Nicotine free base was obtained from this material by dissolving the salt in 1 *N* sodium hydroxide and extracting with dichloromethane, followed by removal of the solvent under reduced pressure.

Chromatography

All separations involved the use of a programmable HPLC system, comprising two Altex Model 110A solvent-metering pumps, an Altex Model 420 system controller and an Altex Model 153 analytical UV detector (254 nm) (Altex, Berkeley, CA, U.S.A.). Two different columns were used; a Partisil PXS SCX 10/25 cation-exchange column, and a Partisil PXS ODS 10/25 reversed-phase column. Both were

obtained from Whatman (Clifton, NJ, U.S.A.). In all cases, samples were introduced via a Rheodyne loop injector (Cotati, CA, U.S.A.) equipped with a 20 μl loop, and UV output was recorded on an Omniscribe Model 5000 dual channel recorder (Houston Instruments, Austin, TX, U.S.A.).

Radioactivity in the column effluent was determined in one of two ways. (a) Directly, by the use of a Model HS Flo-1 radioactive flow-through detector (Radiomatic, Tampa, FA, U.S.A.), equipped with a Radiomatic Model ES stream-splitter. In such cases the scintillation cocktail used was Flo-Scint III, with a mixing ratio of 4 parts to 1 of effluent, by volume. The output of the detector was recorded simultaneously on the second channel of the recorder. (b) Indirectly, by collecting fractions into scintillation vials at one minute intervals, using a Superac Fraction Collector (LKB, Baltimore, MD, U.S.A.) and adding 10 ml of 3a70B cocktail to each vial. In this case ^{14}C activity was measured using a Packard Tri-Carb Scintillation Counter.

All buffers were adjusted to the desired pH after preparation and were filtered and degassed in vacuo prior to use. For the composition of individual mobile phase, see figure legends and Table I.

RESULTS AND DISCUSSION

Variation in enantiomeric ratio of cold standard

Initially, [^{14}C-NH_3]-(±)-nicotine-(+)-bitartrate was injected onto the cation-exchange column alone (*i.e.* without any form of unlabelled standard). In this case the UV absorption was too low to detect, but the ^{14}C activity could be easily observed. The resulting radiochromatogram showed that the ^{14}C activity resided entirely in one peak (Fig. 1a).

When unlabelled (−)-nicotine (2 μg) was coinjected as a standard with the above radioactive material, the UV absorbance was sufficient to produce a peak at the characteristic retention time of cold nicotine. However, the observed radioactivity was now divided into two distinct peaks of almost identical area; one peak eluting at an idential retention time to the cold standard, and one considerably later (Fig. 1b). The maximum observed resolution was 1.49, and at this point the ratio of ^{14}C activity in the two peaks approached 1:1. A similar experiment was then performed, using (+)-nicotine as the cold standard. Again the same characteristic splitting of the ^{14}C activity was observed (Fig. 1c).

In light of these results, authentic racemic nicotine was coinjected with the radioactive material, and the effect on the ^{14}C activity in the eluate was assessed. As evidenced by the data in Fig. 1d, a single UV peak was observed. However, the ^{14}C activity was now confined to a single peak, co-eluting with the cold racemic standard.

The results up to this point had indicated that some degree of excess of one enantiomer of cold nicotine was required to produce the observed radioactive peak-splitting phenomenon. Therefore the ratio of (−)- and (+)-isomers in the cold standard was varied by mixing (−)-nicotine with racemic nicotine. The results of the HPLC analyses are shown in Fig. 2. As can be seen, a slight enantiomeric excess in the cold standard gives rise to partial splitting of the ^{14}C activity. As the proportion of cold (−)-nicotine is increased, the extent of peak-splitting is also increased.

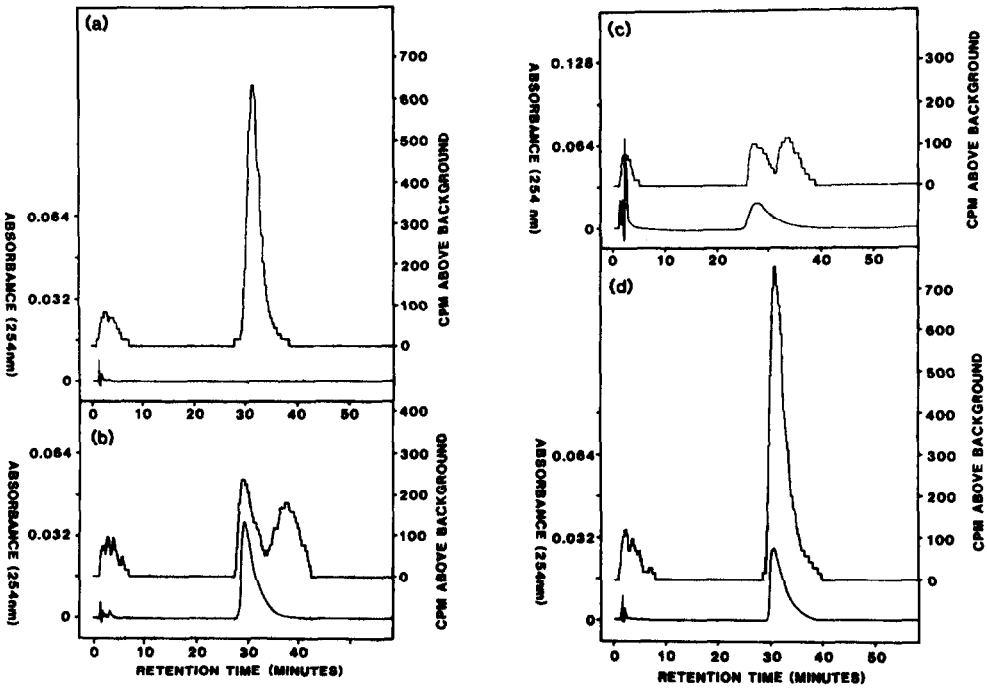


Fig. 1. Radiochromatograms obtained on Partisil-PXS cation-exchange column for [^{14}C-NCH$_3$]-(±)-nicotine-(+)-bitartrate (15 μl, 1 μCi/ml): (a) alone; (b) with unlabelled (−)-nicotine (5 μl, 0.4 mg/ml); (c) with unlabelled (+)-nicotine (5 μl, 0.4 mg/ml); (d) with unlabelled racemic nicotine (5 μl, 0.4 mg/ml). Eluent: 0.06 *M* sodium acetate–methanol (70:30), pH 6.8, 2.0 ml/min. Detection: ^{14}C by Radiomatic Flo-1 HS; UV at 254 nm.

Variation in form of ^{14}C-labelled nicotine

As previously stated, the experiments up to this point had been carried out entirely with [^{14}C-NCH$_3$]-nicotine-(+)-bitartrate. Since the tartrate counterion is optically active, we were not yet able to rule out the possibility of a diastereoisomeric salt effect. Thus (−)-nicotine-(+)-bitartrate and (+)-nicotine-(+)-bitartrate might be expected to exhibit different physical properties, even to the point of chromatographic separation. With this in mind, the effect of varying the enantiomeric ratio of the cold standard was re-examined, using racemic [^{14}C-2′]-nicotine free base as the radiolabelled material.

Again it was observed that one ^{14}C peak was obtained when the radiolabelled material alone was injected onto the cation-exchange system (Fig. 3a). In the presence of cold (−)-nicotine as standard, a splitting phenomenon identical to that seen earlier was produced (Fig. 3b). This simultaneously ruled out the possibilities that either the counterion of the radiolabelled material, or the position of the ^{14}C label were in any way responsible for observed effect.

Since protonation of the pyrrolidine nitrogen produces a second chiral centre, it was also a possibility that internal diastereoisomers may be formed at the pH used in this study. However, it has been shown[25] that protonated nicotine exists to the extent of more than 90% with the methyl group *trans* to the pyridine ring. In this

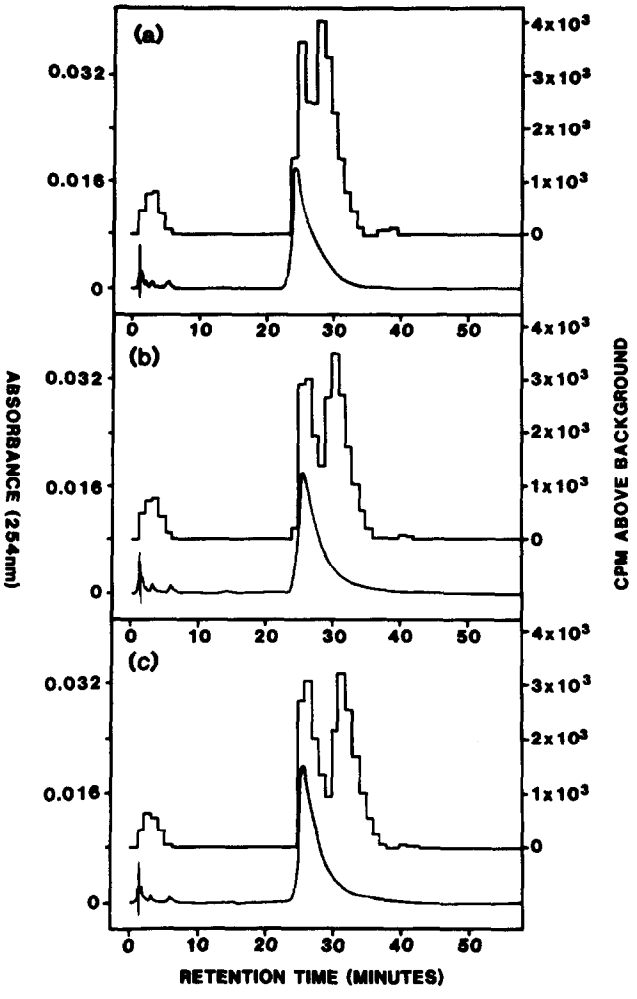


Fig. 2. Effect of variation in enantiomeric ratio of cold standard, using cation-exchange column. [^{14}C-NCH_3]-(±)-nicotine-(+)-bitartrate (15 μl, 1 μCi/ml) coinjected with unlabelled nicotine (5 μl, 0.2 mg/ml) containing: (a) (−)/(±) (1:20); (b) (−)/(±) (1:10); (c) (−)/(±) (1:5). Eluent: 0.12 *M* sodium acetate–methanol (75:25), pH 6.8, 2.0 ml/min. Detection: ^{14}C by scintillation counting of fractions; UV at 254 nm.

way even protonated (*R*)-(+)- and (*S*)-(−)-nicotine are exact mirror images. Therefore, some other phenomenon must be responsible for the peak separation observed in the radiochromatographic analyses described above.

Variation in the HPLC system

In order to ascertain whether the peak-splitting phenomenon was caused by some component of the cation-exchange column or mobile phase, an entirely different HPLC system was developed, using an octadecylsilane (ODS) reversed-phase column. Both forms of ^{14}C-labelled nicotine were analysed on this system, in the presence of cold (−)-nicotine as standard. In both cases, splitting of the ^{14}C activity was

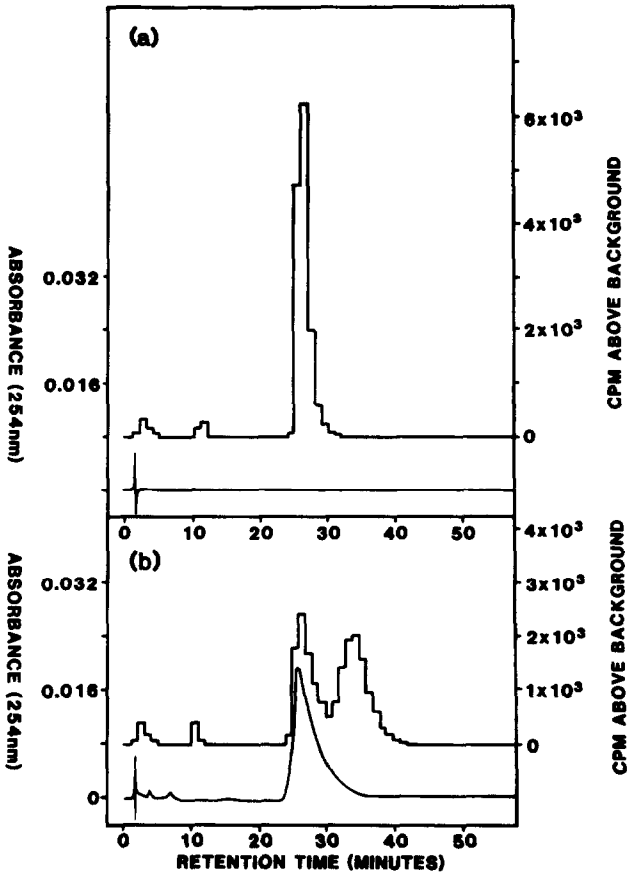


Fig. 3. Effect of variation of form of radiolabelled nicotine, using the cation-exchange column. [^{14}C-2']-(±)-nicotine free base (15 μl, 1 μCi/ml): (a) alone; (b) with unlabelled (−)-nicotine (5 μl, 0.2 mg/ml). Eluent: 0.12 *M* sodium acetate–methanol (75:25), pH 6.8, 2.0 ml/min. Detection: ^{14}C by scintillation counting of fractions; UV at 254 nm.

reproduced (Fig. 4). Using both cation-exchange and ODS columns, a variety of mobile phases were examined for their effect on the separation of [^{14}C-NCH$_3$]-(±)-nicotine-(+)-bitartrate activity. The results of this study are summarized in Table I. In addition, Table I shows the effect of increasing the total ^{14}C activity injected, on the extent of peak splitting. At low levels of radioactivity ($1.5 \cdot 10^{-2}$ μCi), the activity is resolved to the same extent even when increased 4 fold. However, the effect appears to be saturable, since at higher levels (2.3 μCi) the ^{14}C activity elutes as one peak in the presence of cold (−)-nicotine standard.

Variation of the counterion of unlabelled standard

The diastereoisomeric salts (+)-nicotine-di(*p*-toluoyl)-(−)-tartrate and (−)-nicotine-di-(*p*-toluoyl)-(+)-tartrate were prepared by literature methods[10]. Using these salts as cold standards, the splitting of [^{14}C-NCH$_3$]-(±)-nicotine-(+)-bitartrate was examined on the ODS system. For both salts, the ^{14}C activity was again seen to

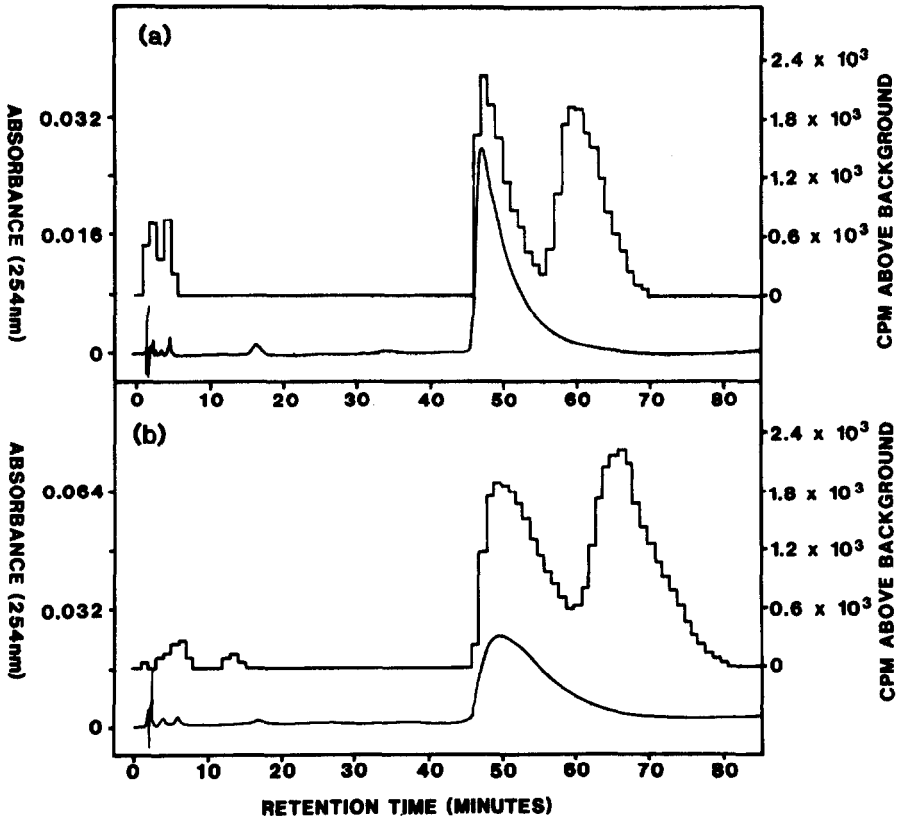


Fig. 4. Effect of variation of HPLC system, using ODS reversed-phase column: (a) [^{14}C-NCH$_3$]-(±)-nicotine-(+)-bitartrate (15 μl, 1 μCi/ml) with unlabelled (−)-nicotine (5 μl, 0.4 mg/ml); (b) [^{14}C-2']-(±)-nicotine (15 μl, 1 μCi/ml) with unlabelled (−)-nicotine (5 μl, 0.4 mg/ml). Eluent: methanol–water (35:65) + 0.2% NH_4OH, pH 7.4, 2.0 ml/min. Detection: ^{14}C by scintillation counting of fractions; UV at 254 nm.

divide into two peaks (Fig. 5a). A similar experiment was performed using the cation-exchange system. Both salts produced the characteristic splitting of ^{14}C activity when coinjected with [^{14}C-NCH$_3$]-(±)-nicotine-(+)-bitartrate (Fig. 5b). Thus it appears that under the conditions of this study, the counterion of the unlabelled nicotine standard has no effect on the radioactive peak-splitting phenomenon. This may be because the large di-(*p*-toluoyl)-tartrate anion becomes dissociated from the protonated nicotinium cation in the mobile phase of the ODS system, or because it is actively separated on the cation-exchange system.

Isolation of ^{14}C peaks by preparative HPLC

Having developed a system which was capable of resolving the two radioactive peaks efficiently, the isolation of the two radioactive components was attempted using preparative reversed-phase HPLC on the analytical ODS column (Fig. 6a, peak A and peak B). Using a fraction collector set at 1-min intervals, the eluate was retained from an injection of unlabelled (*S*)-(−)-nicotine and [^{14}C-NCH$_3$]-(±)-nicotine-

TABLE I

EFFECT OF VARIATION IN MOBILE PHASE, COLUMN PACKING AND TOTAL μCi INJECTED

Column	*Mobile phase*	*Total μCi injected [^{14}C-NCH$_3$] label*	*UV peaks*		*^{14}C Peaks*		*Peak ratio B/A*	*Resolution*
			t'_R (min)	*(%)*	*t'_R (min)*	*(%)*		
SCX	0.06 *M* sodium acetate–methanol (70:30), pH 6.8, 2.0 ml/min	$7.5 \cdot 10^{-3}$	28.5	100	28.5 36.5	46.1 (A) 53.9 (B)	1.17	0.91
SCX	0.12 *M* sodium acetate–methanol (75:25), pH 6.8, 2.0 ml/min	$1 \cdot 10^{-2}$	30.4	100	30.4 45.4	48.3 (A) 51.7 (B)	1.07	1.39
SCX	0.12 *M* sodium acetate–methanol (75:25) pH 6.8, 2.0 ml/min	2.3	24.0	100	24.8	100	–	–
ODS	Methanol–water (45:55) + 0.1% NH_4OH, pH 7.4, 2.0 ml/min	$1.5 \cdot 10^{-2}$	20.2	100	20.6 26.6	40.5 (A) 59.5 (B)	1.46	0.79
ODS	Methanol–water (35:65) + 0.2% NH_4OH, pH 7.4, 2.0 ml/min	$1.5 \cdot 10^{-2}$	48.0	100	48.0 64.0	44.1 (A) 55.9 (B)	1.27	1.03
ODS	Methanol–water (35:65) + 0.2% NH_4OH, pH 7.4, 2.0 ml/min	$6 \cdot 10^{-2}$	46.6	100	47.2 60.4	44.1 (A) 55.9 (B)	1.27	0.99

(+)-bitartrate. A small quantity (10 μl) of each fraction was examined by scintillation counting to determine the ^{14}C content. The fractions within each radioactive peak were then combined and lyophilised, to yield samples of peak A and peak B. Each of these samples was redissolved in the minimum volume of methanol and coinjected with cold (−)-nicotine standard onto the same HPLC system. Peak A again coeluted with the cold standard (Fig. 6b) and peak B eluted after the unlabelled material (Fig. 6c). In neither case was there any evidence of redistribution of the ^{14}C activity.

The immediate implication of this result is that the two peaks contain the separated enantiomers of radiolabelled nicotine. If peak B had contained both (+)- and (−)-radiolabelled isomers, then peak A must have been similarly composed. If that were true, then isolated peaks would be expected to redistribute their activity when reinjected into the same chromatographic system. Similarly if peaks A and B were due to different ionised forms of radiolabelled nicotine, they would each be expected to redistribute on reinjection.

Variation in enantiomeric ratio of ^{14}C-labelled nicotine

As previously stated, both forms of commercially available ^{14}C-labelled nicotine used in this study were racemic. Direct chemical resolution of these samples

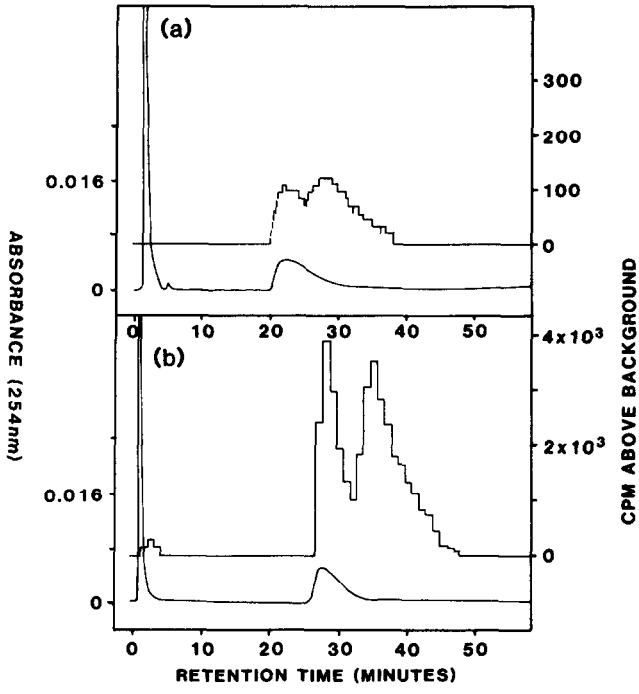


Fig. 5. Effect of counterion of unlabelled nicotine standard, using [^{14}C-NCH$_3$]-(±)-nicotine-(+)-bitartrate (15 μl, 1 μCi/ml) and (−)-nicotine-di-(*p*-toluoyl)-(+)-tartrate (5 μl, 0.25 mg/ml): (a) on ODS column (eluent: methanol–water (35:65) + 0.2% NH_4OH, pH 7.4, 2.0 ml/min. Detection: ^{14}C by Radiomatic Flo-1 HS; UV at 254 nm); (b) on cation-exchange column (eluent: 0.12 *M* sodium acetate–methanol (75:25), pH 6.8, 2.0 ml/min. Detection: ^{14}C by scintillation counting of fractions; UV at 254 nm).

would have been impossible with the quantity of material involved. However, we were able to take advantage of the fact that cold (*S*)-(−)-nicotine must be added eventually to any radiolabelled sample to determine the extent of ^{14}C splitting. In view of this, a small quantity of radiolabelled material was resolved in the presence of excess cold (−)-nicotine.

Di-(*p*-toluoyl)-(+)-tartaric acid (202 mg, 0.5 mmol) was dissolved in 1.0 ml of absolute ethanol. To this stirred solution was added a mixture of cold (−)-nicotine (81 mg, 0.5 mmol) and 5 μCi of [^{14}C-2']-(±)-nicotine. The crystalline product was filtered and washed with ethanol, and the filtrate and crystals retained. Di(*p*-toluoyl)-(+)-tartaric acid preferentially forms a diastereoisomeric salt with (−)-nicotine. Since (−)-nicotine was present in excess, the radiolabelled (−)-nicotine would be almost entirely distributed into the crystalline product (any remaining in the mother liquor would have undergone a 6000 fold reduction in specific activity). Conversely, the radiolabelled (+)-nicotine would be almost entirely in the mother liquor. This effect is summarized in Scheme 1.

Thus, the above crystalline product was reconverted to the free base by the method previously stated, and a methanolic solution was injected directly onto the cation-exchange column. As shown by Fig. 7, the remaining ^{14}C activity in the resulting radiochromatogram was almost completely confined to one peak, coeluting

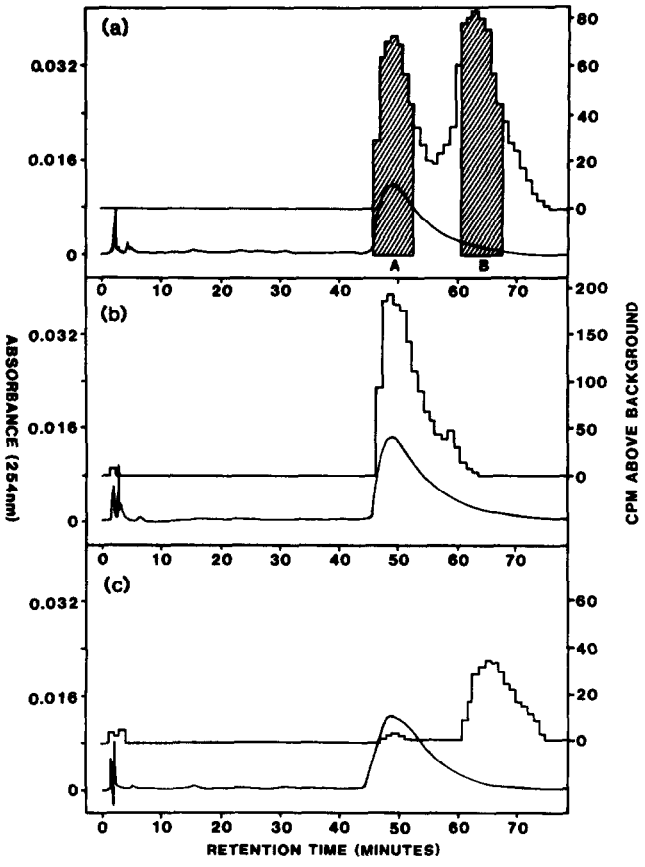
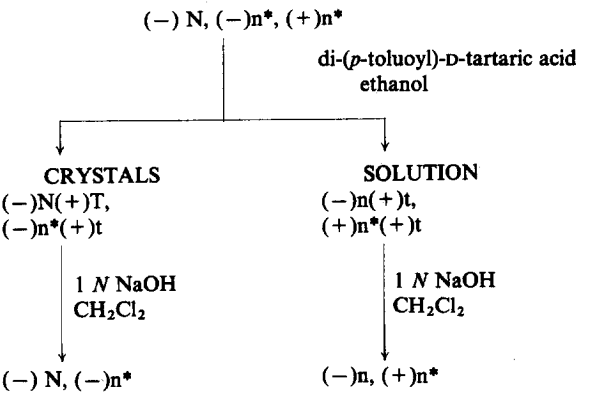


Fig. 6. Isolation of radioactive peaks A and B, using [^{14}C-NCH_3]-(±)-nicotine-(+)-bitartrate (15 μl, 4.0 μCi/ml) and unlabelled (−)-nicotine (5 μl, 0.4 mg/ml) on the ODS column. (a) Radiochromatogram from scintillation counting of 10 μl of each 1 min fraction; (b) peak A (from, recombined fractions) reinjected with cold (−)-nicotine (5 μl, 0.2 mg/ml); peak B (from recombined fractions) reinjected with cold (−)-nicotine. Eluent: methanol–water (35:65) + 0.2% NH_4OH, pH 7.4, 2.0 ml/min. Detection: ^{14}C by scintillation counting of fractions; UV at 254 nm.

with the cold (−)-nicotine, which was present in excess. Thus radiolabelled (−)-nicotine must elute in peak A when cold (−)-nicotine is used as the standard.

The filtrate obtained from the above resolution was also reconverted to free base, and a methanolic solution was injected directly onto the cation-exchange column (Fig. 8a). It was not necessary to add cold standard to this sample, as (−)-nicotine was already present. In this case the ^{14}C activity was entirely confined to peak B in the resulting radiochromatogram, eluting after the UV peak of the cold material. Thus radiolabelled (+)-nicotine must elute in peak B when cold (−)-nicotine is the standard.

It therefore appears that an excess of one enantiomer of nicotine must induce a difference in the retention behavior of the two enantiomers of radiolabelled nicotine. This appears to be a saturable phenomenon; separation of nanogram quantities of radiolabelled material is easily observed in the radiochromatograms obtained, but



Scheme 1. Resolution of radiolabelled nicotine in presence of excess (*S*)-(−)-nicotine. N,n are nicotine (N ≫ n) and T,t are di-(*p*-toluoyl)-D-tartrate (T ≫ t). * Indicates ^{14}C label.

much larger quantities of unlabelled material are required to give an appreciable UV absorbance. Thus separation of cold enantiomers cannot be observed without saturation. However, this does not preclude the application of this method to the resolution of racemic radiolabelled materials, as the amount of material involved is usually very small.

Using the sample prepared from the mother liquor of the above resolution [containing radiolabelled (+)-nicotine and cold (−)-nicotine], the effect of adding cold (+)-nicotine on the position of the single ^{14}C peak was examined using the cation-exchange system. As shown in Fig. 8, addition of 50 ng of cold (+)-nicotine causes a redistribution of the ^{14}C activity in the radiochromatogram, so that a small proportion coelutes with the cold standard. When sufficient cold (+)-nicotine has

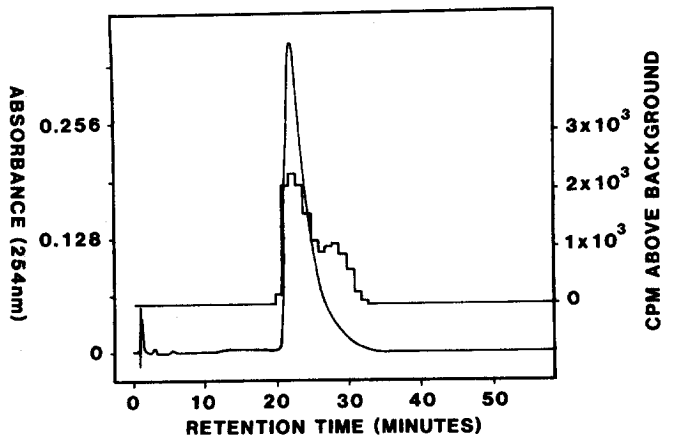


Fig. 7. Effect of variation of enantiomeric composition of labelled material. Radiochromatogram obtained for the free base generated from the crystalline product of reaction of cold (−)-nicotine with di-(*p*-toluoyl)-(+)-tartaric acid in the presence of [^{14}C-2′]-(±)-nicotine. Column: cation-exchange. Eluent: 0.12 *M* sodium acetate–methanol (75:25), pH 6.8, 2.0 ml/min. Detection: ^{14}C by scintillation counting of fractions; UV at 254 nm.

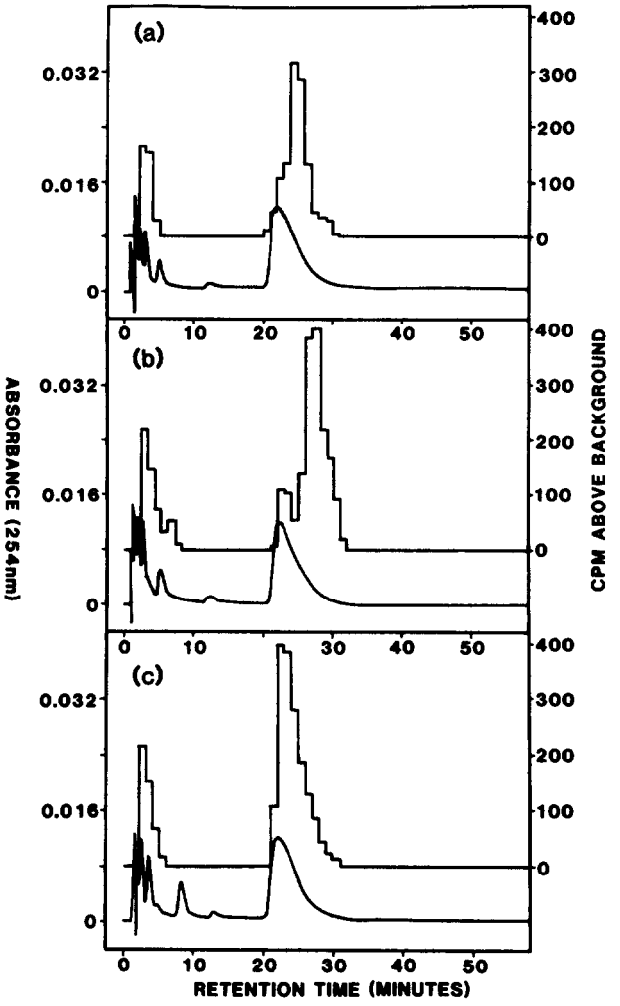


Fig. 8. Effect of adding cold (+)-nicotine to a mixture of cold (−)-nicotine and [^{14}C-2']-(+)-nicotine. Radiochromatograms of the free base generated from the mother liquor of the reaction of cold (−)-nicotine with di-(*p*-toluoyl)-(+)-tartaric acid in presence of [^{14}C-2']-(±)-nicotine. (a) Alone; (b) after addition of 50 ng of cold (+)-nicotine; (c) after addition of 2 μg of cold (+)-nicotine. Column: cation-exchange. Eluent: 0.12 *M* sodium acetate–methanol (75:25), pH 6.8, 2.0 ml/min. Detection: ^{14}C by scintillation counting of fractions; UV at 254 nm.

been added to provide a near-racemic composition, all of the ^{14}C activity in the sample elutes with the cold standard.

Precedents for the observed phenomenon

Despite the widely held belief that optical isomers are chemically identical, there is some precedent in the literature for the phenomenon we have observed. A number of investigators[26–28] have reported a difference between the nuclear magnetic resonance (NMR) spectra of racemates and pure enantiomers of certain optically active compounds, *e.g.* dihydroquinine[28]. Whenever there is an excess of one enan-

(a) $(-)^* + (-) \rightleftharpoons (-)^*(-)$ *homo*-dimer peak A

$(+)^* + (-) \rightleftharpoons (+)^*(-)$ *hetero*-dimer peak B

(b) $(+)^* + (+) \rightleftharpoons (+)^*(+)$ *homo*-dimer peak A

$(-)^* + (+) \rightleftharpoons (-)^*(+)$ *hetero*-dimer peak B

Scheme 2. (a) Association of labelled nicotine in excess cold (*S*)-(−)-nicotine. (b) Association of labelled nicotine in excess cold (*R*)-(+)-nicotine. * Indicates ^{14}C label.

tiomer in a sample, the NMR spectrum splits; smaller signals appear at different chemical shift values for the lesser of the two isomers, with a relative intensity corresponding to the proportion of that enantiomer in the mixture. As the sample approaches racemic composition, the signals of each enantiomer become equal in intensity and superimpose. This unusual behavior is analogous to the phenomenon observed for ^{14}C-nicotine in the present study. One possible explanation is that nicotine exists predominantly in an associated form in solution. This may be oligomeric, but is best illustrated by consideration of a dimeric association. An association between two like enantiomers will produce a dimer with slightly different properties to one composed of unlike enantiomers. The thermodynamics of these interactions may or may not be the same, but the proportion of *homo*- or *hetero*-dimers would certainly be under statistical control. Thus, with an excess of (−)-nicotine, both isomers of the radiolabelled material would be predominantly associated with the cold material, as shown in Scheme 2(a). In this way the activity due to radiolabelled (+)-nicotine would be entirely associated in *hetero*-dimers, whereas that due to radiolabelled (−)-nicotine would be entirely in *homo*-dimers. If the two dimers were sufficiently different to produce unequal affinities for the column/mobile phase then separation of the two radiolabelled enantiomers could be achieved by HPLC. The converse applies when (+)-nicotine is the cold standard; activity due to radiolabelled (+)-nicotine would be predominantly in *homo*-dimers, and that due to (−)-nicotine in *hetero*-dimers [Scheme 2(b)]. The behaviour of radiolabelled nicotine when injected alone, or with cold racemic standard, suggests that *homo*-association is preferred to *hetero*-association, unless precluded by concentration effects. Thus, in both cases *homo*-dimers alone are formed. As the proportion of enantiomers in the cold standard is varied from optically pure towards racemic composition, the probability of forming *homo*-dimers increases, until all the ^{14}C activity elutes as one peak.

It must be emphasized that the above explanation of the phenomenon is purely speculative, since there is no evidence to suggest that nicotine associates in solution. Thus the nature of the chromatographic behavior exhibited by radiolabelled nicotine isomers remains to be established. At the pH's used in this study, nicotine is predominantly protonated on the 1′-N atom, thus hydrogen bonding may play a significant role. The analogous NMR phenomenon discussed above has only been observed in non-polar solvents. However, the on-column environment may be such that enantiomeric association is favoured.

The apparent saturability of the observed phenomenon is more difficult to explain. Unlabelled (±)-nicotine does not show peak splitting, as the amount of

material required to produce an observable UV absorption is sufficient to cause saturation. Thus it appears that, based upon our hypothesis, the ability of the column to distinguish between *homo-* and *hetero*-dimers may be limited. Alternatively, saturation may result from solvation effects; small amounts of *hetero*-dimer may have a reduced affinity for a mobile phase which is predominantly composed of solvated *homo*-dimers. This reduced affinity may be overcome when there is sufficient *hetero*-dimeric material present to promote its own solvation. This is analogous to the principle of fractional crystallization, *i.e.* the predominant disastereoisomer crystallizes to the exclusion of the lesser. If the solution is saturated, both disastereoisomers will crystallize.

CONCLUSIONS

It is immediately apparent that the phenomenon reported in this study is of potential value in the separation of the enantiomers of ^{14}C-labelled nicotine. In the presence of unlabelled (−)-nicotine, the ^{14}C activity in the radiochromatogram obtained from HPLC analysis is split in such a way that the second peak contains only the (+)-radiolabelled isomer. If, as we propose, the splitting of the activity is due to differential association of the two radiolabelled enantiomers with the cold standard,

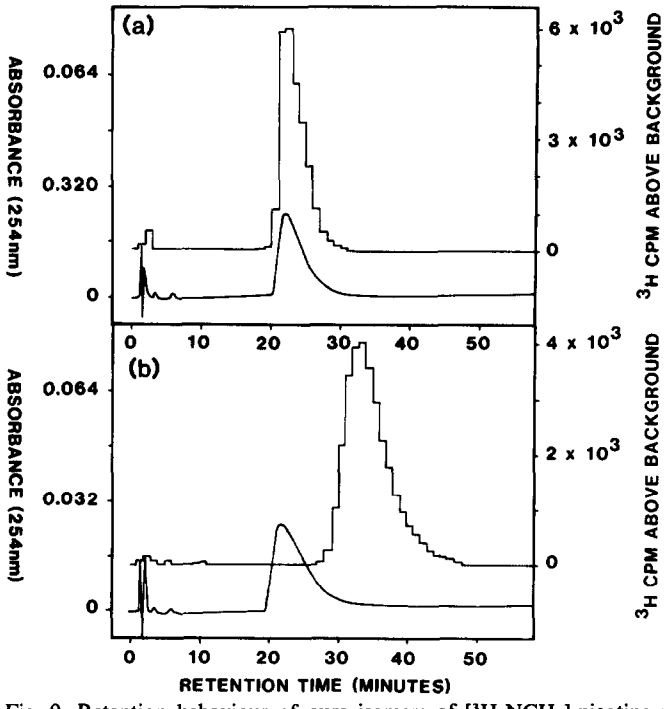


Fig. 9. Retention behaviour of pure isomers of [^{3}H-NCH$_3$]-nicotine when coinjected with unlabelled (−)-nicotine: (a) unlabelled (−)-nicotine (5 µl, 0.4 mg/ml) with [^{3}H-NCH$_3$]-(−)-nicotine (15 µl, 1 µCi/ml); (b) unlabelled (−)-nicotine (5 µl, 0.4 mg/ml) with [^{3}H-NCH$_3$]-(+)-nicotine (15 µl, 1 µCi/ml). Column: cation-exchange. Eluent: 0.12 *M* sodium acetate–methanol (75:25), pH 6.8, 2.0 ml/min. Detection: ^{3}H by scintillation counting of fractions; UV at 254 nm.

then the second peak may also contain a small amount of unlabelled (−)-nicotine. However, all ^{14}C activity (> 99%) would be due to the radiolabelled (+)-enantiomer. Thus if this material is isolated it may be used for metabolic or receptor binding studies. The first peak, eluting with the cold standard would be radiochemically pure (−)-nicotine, but would have a much reduced specific activity because of dilution by cold material. However, by using cold (+)-nicotine as the standard instead of its antipode, the second of the two ^{14}C peaks produced would contain only the (−)-radiolabelled enantiomer, undiluted by cold (−)-nicotine.

We have recently obtained samples of [^{3}H-NCH_3]-nicotine from NEN (Boston, MA, U.S.A.) which are claimed to be the pure optical isomers of the radiolabelled material. [^{3}H-NCH_3]-(−)-nicotine elutes as one radioactive peak when coinjected with unlabelled (*S*)-(−)-nicotine onto the HPLC systems used in this study, with a retention time identical to the cold material (Fig. 9a). [^{3}H-NCH_3]-(+)-nicotine also elutes as a single radioactive peak when coinjected with cold (*S*)-(−)-nicotine onto these systems. However, this single ^{14}C peak has a much greater retention time than the cold standard (Fig. 9b). These results would appear to be further evidence in support of our hypothesis.

Another less obvious application of the observed phenomenon arises from the effect seen when small quantities of cold (+)-nicotine are added to a mixture of cold (−)-nicotine and radiolabelled (+)-nicotine. The ^{14}C peak in the radiochromatogram is gradually displaced, in a manner which reflects the amount of cold (+)-nicotine present (Fig. 8). Therefore, this procedure could potentially be developed into a sensitive method for the detection of small quantities of (+)-nicotine in biological fluids. Using the opposite enantiomers, the technique could also be applied for the detection of small quantities of (−)-nicotine.

The phenomenon of differential enantiomeric association appears to be widespread among optically active compounds[29]. It may also be true that the unusual chromatographic behavior of ^{14}C-labelled nicotine is a more general phenomenon. If this is so, then the methods discussed in this study may be applicable to the resolution of a number of racemic radiolabelled compounds. Further work is at present in progress to investigate this possibility, and to establish the exact nature of the above chromatographic behaviour exhibited by radiolabelled nicotine isomers.

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REFERENCES

1 J. W. Gorrod and P. Jenner, *Essays Toxicol.*, 6 (1975) 35–78.
2 P. Jenner, J. W. Gorrod and A. H. Beckett, *Xenobiotica*, 3 (1973) 563–572.
3 P. Jenner, J. W. Gorrod and A. H. Beckett, *Xenobiotica*, 3 (1973) 573–580.
4 W. M. Moore and R. N. Brady, *Biochim. Biophys. Acta*, 441 (1976) 252–260.
5 V. A. Eterovic and E. L. Bennett, *Biochim. Biophys. Acta*, 362 (1974) 346–355.
6 M. T. Alderice and G. B. Weiss, *Arch. Int. Pharmacodyn. Ther.*, 218 (1975) 252–267.

7 L. G. Abood, K. Lowy, A. Tometsko and H. Booth, *J. Neurosci. Res.*, 3 (1978) 327–333.
8 M. D. Aceto, B. R. Martin, I. M. Uwaydah, E. L. May, L. S. Harris, C. Izazole-Conde, H. L. Dewey, T. J. Bradshaw and W. C. Vincek, *J. Med. Chem.*, 22 (1979) 174–177.
9 M. Nakane and C. R. Hutchinson, *J. Org. Chem.*, 43 (1980) 3922–3931.
10 E. R. Bowman, H. McKennis, Jr., and B. R. Martin, *Synthetic Communications*, 12 (1982) 871–879.
11 W. C. Vincek, B. R. Martin, M. D. Aceto and E. R. Bowman, *J. Med. Chem.*, 23 (1980) 960–962.
12 W. C. Vincek, B. R. Martin, M. D. Aceto, H. L. Tripathi, E. L. May and L. S. Harris, *J. Pharm. Sci.*, 70 (1981) 1292–1293.
13 E. Leete, H. V. Isaacson and H. D. Durst, *J. Labelled Compd.*, 7 (1971) 313–317.
14 R. A. Comes, M. T. Core, M. D. Edmonds, W. B. Edwards, and R. W. Jenkins, *J. Labelled Compd.*, 9 (1973) 253–259.
15 P. Jacob, *J. Org. Chem.*, 47 (1982) 4165–4167.
16 E. Hansson and C. G. Schmiterlow, *J. Pharmacol. Exp. Ther.*, 137 (1962) 91–102.
17 C. G. Schmiterlow and E. Hansson, *Nature (London)*, 194 (1962) 298–299.
18 H. McKennis, Jr., L. B. Turnbull and E. R. Bowman, *J. Biol. Chem.*, 238 (1963) 719–723.
19 K. Decker and R. Sammeck, *Dtsch. Med. Forsch.*, 2 (1964) 33–36.
20 L. E. Appelgren, E. Hansson and C. G. Schmiterlow, *Acta. Physiol. Scand.*, 56 (1962) 249–257.
21 G. Anderson, E. Hansson and C. G. Schmiterlow, *Experientia*, 21 (1965) 211–213.
22 H. McKennis, Jr., E. Wada, E. R. Bowman and L. B. Turnbull, *Nature (London)*, 190 (1961) 910–911.
23 I. S. Krull, *Adv. Chromatogr.*, 16 (1978) 175–210.
24 K. C. Cundy and P. A. Crooks, *J. Chromatogr.*, in press.
25 I. Whidby and J. I. Seeman, *J. Org. Chem.*, 41 (1976) 1585–1590.
26 A. Horeau and J. P. Guette, *Tetrahedron*, 30 (1974) 1923–1931.
27 M. I. Kabachnik, T. A. Mastryukova, E. I. Fedin, M. S. Vaisberg, L. L. Morozov, P. V. Petrosky and A. E. Shipov, *Tetrahedron*, 32 (1976) 1719–1728.
28 T. Williams, R. G. Pitcher, P. Bommer, J. Gutzwiller and M. Uskokovic, *J. Am. Chem. Soc.*, 91 (1969) 1871–1872.
29 W. H. Pirkle and D. J. Hoover, *Top. Stereochem.*, 13 (1982) 263–331.