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Short communication

Methylated *N*ε-dansyl-L-lysine as a fluorogenic reagent for the chiral separation of carboxylic acids

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Abstract

Dansyl amino acids having a free amino group and an asymmetric carbon atom were examined as a labeling reagent for chiral compounds containing carboxylic moieties to realize enantiomeric separation as well as fluorimetric determination. We tested dansyllysine by reacting it with (+)- and (−)-ibuprofen as a model carboxylic enantiomer. As the intramolecular carboxylic moiety of the dansyl amino acid interfered in the condensation reaction to form an amide bond with the carboxylic acid, the moiety was masked by methylation with trimethylsilyldiazomethane before the reaction. These derivatives were reacted with (+)- and (−)-ibuprofen and better enantiomeric resolution was achieved with methylated dansyllysine on a reversed-phase column. The derivatisation reaction was facilitated by the use of catalysts that are commonly employed in peptides synthesis. The reaction was completed within 5 min at room temperature when diethyl phosphorocyanidate was used. Due to the dansyl moiety, methylated dansyl-lysine enables a sensitive determination of ibuprofen with a fluorescence detector, in addition to the capability of enantiomer resolution. In tests, the detection limits for (+) and (−)-ibuprofen were 4 pmol per injection ($S/N=3$) at an excitation wavelength of 340 nm and an emission wavelength of 523 nm. Linear responses for the determination of (+) and (−)-ibuprofen in human urine were also demonstrated ($r \geq 0.998$) in the range from 10 to 1000 ng/ml. The precision and accuracy for urine samples spiked with (+)- and (−)-ibuprofen at 10, 100 and 1000 ng/ml were <10.1 and <14.6% ($n=4$), respectively.

Keywords: Enantiomer separation; *N*ε-Dansyl-L-lysine; Carboxylic acids

1. Introduction

Many compounds that exhibit biological and pharmacological activities have carboxylic moieties and they sometimes contain chiral atoms. Differences in the toxicological action and pharmacokinetic behavior of enantiomers in the body are widely recognized, as are differences in their biological and pharmacological potency. Interconversion of each enantiomer after drug administration is also of concern, even when a single enantiomer is administered. Thus, a method that is capable of determining each enantiomer and of tracing dynamic changes or interconversion of racemates in organisms is needed for pharmaceutical development. Many derivatisation reagents for carboxylic acids

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have been developed and applied. Mayer et al. [1] and Takasaki and Tanaka [2] have reported stereoselective determination methods for carboxylic acids using fluorescent chiral coupling reagents. These reagents have a benzoxazole or naphthyl group as a fluorophore, respectively. Conversely, reagents with dansyl structures have widely been accepted for use in high-performance liquid chromatography (HPLC) assays due to their strong fluorescence. Monodansylcadaverine (MDC) [3] and *N*-bromoacetyl-*N′*-dansylpiperazine [4] are representative reagents. However, they are essentially intended for non-enantiospecific analysis. 1-(4-Dansylaminophenyl)ethylamine [5] and 6-methoxy-2-(4-substituted phenyl)benzoxazole [6] were designed to provide enantiomeric resolution. These reagents, however, require heating and/or a long incubation time to complete the derivatisation reaction. Moreover, these reagents need to be synthesized and purified in the laboratory. MDC appeared to have attractive reactivity under mild conditions [3,7], in addition to strong fluorescence. Therefore, we have searched for a new labeling reagent that is ready-to-use, has strong fluorescence like MDC but has an additional function for chiral separation. Based on this concept, dansyl derivatives of amino acids were examined. In this paper, we show that methylated dansyllysine has favorable characteristics, contributing to a rapid reaction under mild conditions and enantiomeric resolution, ease of use and strong fluorescence for chiral carboxylic compounds.

2. Experimental

2.1. Reagents

All reagents used in this study were of analytical-reagent grade unless otherwise stated. *Nε*-Dansyl-L-lysine (enantiomeric purity, 99%) was purchased from Sigma (St. Louis, MO, USA). *S*(+)- and *R*(−)-ibuprofen (IBP; enantiomeric purity, 99.95 and 97.90%, respectively) were from Funakoshi (Tokyo, Japan). Triphenylphosphine (TPP), 1-hydroxybenzotriazole (HOBt), dicyclohexylcarbodiimide (DCC) and diethyl phosphorocyanidate (DEPC) were obtained from Wako (Osaka, Japan). 2,2′-Dipyridyl disulfide (DPDS), diphenylphosphoryl azide (DPPA) and trimethylsilyldiazomethane (TMSDM) were from Tokyo Kasei (Tokyo, Japan). Acetonitrile was of HPLC grade and was supplied from Kanto (Tokyo, Japan). Water, produced by a Milli-Q SP TOC system with a Labo Ionpure 12 apparatus (Nihon Millipore, Tokyo, Japan), was used throughout.

2.2. Apparatus

The HPLC system consisted of an SCL-6B system controller, a SIL-6B autoinjector, two LC-10AS pumps (all from Shimadzu), an 820-FP spectrofluorometer (Jasco, Tokyo, Japan), an SSC column oven 3512C (Senshu Scientific, Tokyo, Japan) and a Shimadzu RF-5000 spectrofluorophotometer (Kyoto, Japan). Chromatographic data were collected and processed by an Access*Chrom chromatographic data system (Perkin Elmer Japan, Yokohama, Japan).

2.3. Methylation of dansyl amino acids

The carboxyl group of the dansyl amino acid was methylated with TMSDM according to the method reported by Hashimoto et al. [8]. In brief, 53.2 mg of dansyl amino acid were dissolved in methanol (2 ml) and then benzene (8 ml) was added, followed by the addition of TMSDM (0.4 ml, 10% hexane solution). The mixture was stirred for 30 min at room temperature and was evaporated to dryness under a stream of nitrogen at 40°C. The residue was reconstituted in 5 ml of methanol in order to give a final concentration of 28 m*M* and was stored at 4°C until required for the enantiomer derivatisation reaction.

2.4. Derivatisation procedure

A 50-μl volume of an IBP–methanol solution was dispensed into a glass tube, and 100 μl of a methylated dansyl (Dns)-amino acid solution (28 m*M*), prepared as described in Section 2.3, was added. After adding 10 μl of DEPC, the mixture was allowed to stand for 5 min at room temperature. A 40-μl volume of water was added to terminate the reaction and a 10-μl aliquot of the resulting solution was subjected directly to HPLC analysis.

2.5. Extraction procedure for spiked urine samples

To 1 ml of an IBP-spiked urine sample, 1 ml of 100 m*M* acetate (pH 2.0) was added. After mixing, the urine sample was loaded onto an Isolute HAX cartridge column (International Sorbent Technology, Mid Glamorgan, UK), which had been preconditioned with 3 ml of methanol, water and 100 m*M* acetate (pH 2.0), in turn. After the column was washed with 2 ml each of water, 40% aqueous methanol, hexane containing 10% triethylamine and hexane, the charged IBP was eluted with 1 ml of methanol containing 10 m*M* hydrochloric acid. The eluate was evaporated to dryness under a stream of nitrogen at 50°C. The residue was reconstituted with 50 μl of methanol and derivatisation was done with methylated Dns-Lys as described above.

2.6. Chromatographic conditions

Resolution of (±)ibuprofen methylated Dns-Lys derivatives was achieved on an L-column ODS (5 μm, 120 Å, 250×4.6 mm I.D., Chemicals Inspection and Testing Institute, Tokyo, Japan), which was maintained at 35°C. Gradient elution was carried out by linearly changing the acetonitrile composition from 65% (v/v) to 80% in water at a flow-rate of 1.0 ml/min over 20 min. Detection was accomplished by monitoring the fluorescence attributable to the dansyl moiety (excitation, 340 nm; emission, 523 nm).

3. Results and discussion

3.1. Selection of dansyl amino acids as a derivatisation reagent for carboxylic acid

The reactivity of dansyl amino acids having a free amino functional group such as Dns-Lys with IBP that was selected as a model compound having a carboxyl moiety was studied. The dansyl amino acid did not react with IBP even in the presence of a catalyst, DEPC. The carboxylic groups of the dansyl amino acid presumably interfered in the condensation reaction with carboxylic acids. Therefore, before the reaction, the intramolecular carboxylic moiety was masked with TMSDM (Fig. 1a), which is a well-known methylation reagent [9] whose utility was proved previously [10]. After masking the carboxylic moiety of the dansyl amino acid, derivatisation of IBP was carried out. Methylated Dns-Lys gave chromatographic peaks corresponding to the products with IBP and yielded a higher fluorescence intensity.

3.2. Optimization of the derivatisation conditions

Based on the reaction mechanism of MDC [3,7], we first examined the effect of reaction solvent with dimethylformamide, dimethyl sulfoxide, tetrahydrofuran (THF), methanol and acetonitrile on the derivatisation reaction. The derivatisation reaction proceeded quicker and gave a larger peak response with all of the solvents, except for THF at ambient temperature (Fig. 2). Methanol was selected as a reaction solvent because of its ease of handling. Secondly, representative catalysts for the condensation reaction, i.e., DEPC [11], DCC–HOBt [12], DPDS–TPP [13] and DPPA [14], were tested and the reaction was facilitated by DEPC (Fig. 3). Thirdly, the concentration of methylated Dns-Lys that was reacted with 500 nmol of IBP was varied; 28 m*M* methylated Dns-Lys was found to be sufficient. Under these conditions, the derivatisation reaction was optimized by changing the reaction temperature and time. The reaction was completed within 5 min at room temperature (ca. 25°C) (Fig. 1b). In tests, methylated Dns-Lys was stable for two weeks at 4°C. The IBP derivative with methylated Dns-Lys was stable for at least 48 h.

3.3. Separation of (±)-IBP derivatives and quantification

Using a standard solution and a spiked urine sample with (+)- and (−)-IBP, separation and quantification were investigated. A typical chromatogram is shown in Fig. 4 and illustrates the separation of (+)- and (−)-IBP on an ODS column within 20 min by gradient elution with aqueous acetonitrile, as described in Section 2.

Although we carried out the same experiment with another dansyl amino acid, *O*-dansyl-L-tyrosine, the resolution of (±)-IBP derivatives with methylated Dns-Lys was superior to that with methylated Dns-Tyr (Fig. 5). Having considered the resolution and

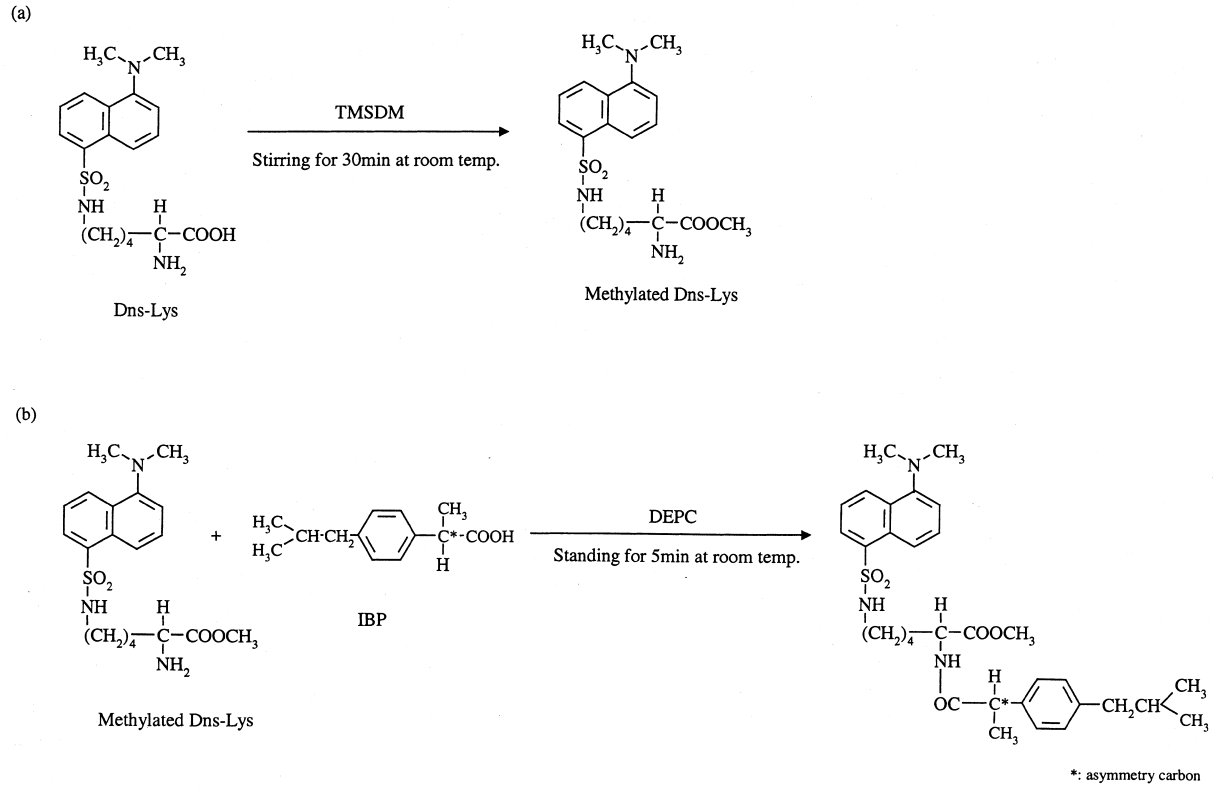

Fig. 1. Scheme for the synthesis of the chiral derivatisation reagent (a) and for the derivatisation of ibuprofen with chiral derivatisation reagents (b). TMSDM, trimethylsilyldiazomethane; DEPC, diethyl phosphorocyanidate.

fluorescence yield, it was found that methylated Dns-Lys was a superior fluorescence–chiral derivatisation reagent compared with methylated Dns-Tyr. The fluorescence intensity of the two diastereomer derivatives was the same. For quantification, calibration graphs were plotted with the peak area of both (+)- and (−)-IBP labeled with methylated Dns-Lys and linear responses ($r \geq 0.999$) were obtained in the range from 2 to 200 ng per injection (10 μl). The repeatability at 20 ng per injection was 1.64% ($n=6$). The detection limit was 800 pg per injection (10 μl), calculated at a signal-to-noise ratio of three.

The methylated Dns-Lys was also used for the enantiomeric determination of IBP in human urine. Blank human urine was spiked with (+)- and (−)-IBP and extracted using a solid-phase cartridge. The extracts were evaporated and derivatised with methylated Dns-Lys. A typical chromatogram is shown in Fig. 6. Linear responses ($r \geq 0.998$) were obtained for both enantiomers in the range from 10 to 1000 ng/ml in urine. The precision and accuracy at 10, 100 and 1000 ng/ml were <10.1 and <14.6% ($n=4$), respectively.

As demonstrated with IBP enantiomers, methylated Dns-Lys is a potent and facile derivatisation reagent for carboxylic compounds in terms of reactivity and sensitive quantification by fluorescence. It also provides enantiomeric separation if target carboxylic acids have chiral centers. The methylated Dns-Lys could be used for the determination of extracted enantiomeric drug from urine. Methylated Dns-Lys is prepared by reacting dansyllysine with TMSDM for 30 min at ambient temperature. In contrast, many derivatisation reagents [1,2,4–6] for chiral and achiral carboxylic acids had to be synthesized and purified by complicated procedures before use. Moreover, the methylated Dns-Lys derivatisation reaction is completed in a short time, e.g.

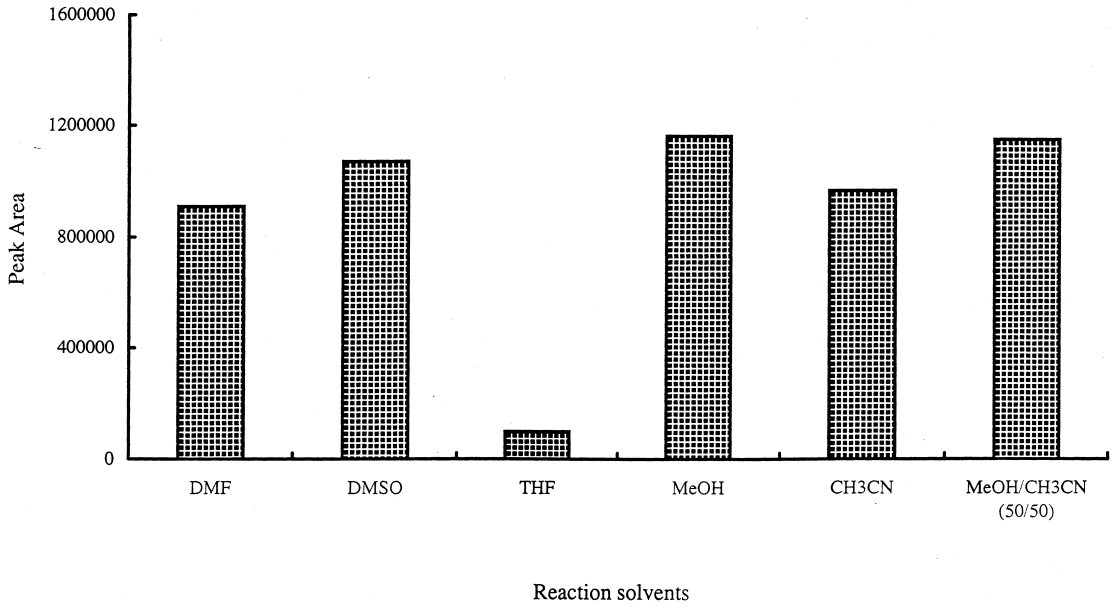

Fig. 2. Effect of reaction solvents on the derivatisation reaction of ibuprofen with methylated dansyllysine. A constant amount of ibuprofen (500 nmol) was derivatised with constant amounts of different solvents (100 μl) in the presence of a constant amount of methylated Dns-Lys (28 m*M*) and DEPC (10 μl). Dns-Lys, dansyllysine; DEPC, diethyl phosphorocyanidate.

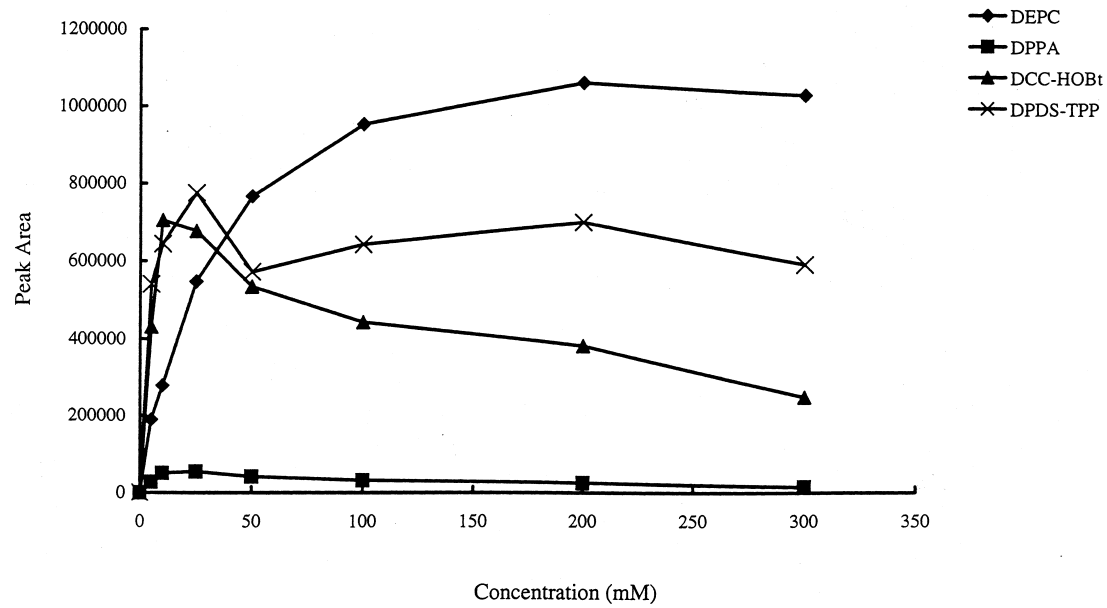

Fig. 3. Effect of the concentration of catalyst on the derivatisation reaction of ibuprofen with methylated dansyllysine. A constant amount of ibuprofen (500 nmol) was derivatised with a constant amount of methylated dansyllysine (28 m*M*) in the presence of different amounts of different catalysts (5–300 m*M*).

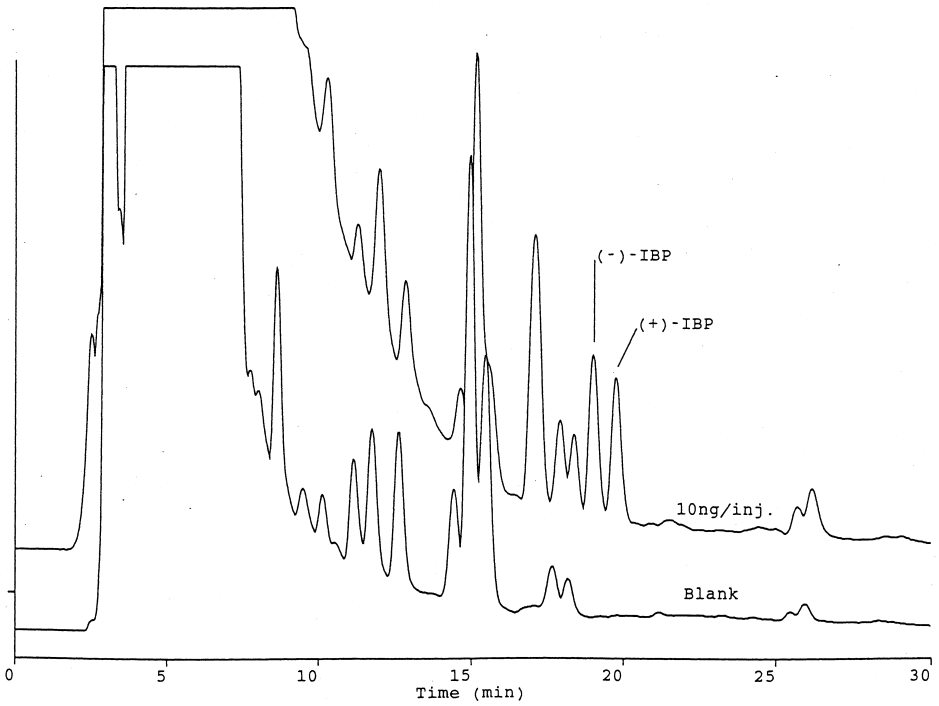

Fig. 4. Chromatograms of methylated dansyllysine derivatives of ibuprofen. HPLC conditions were the same as those listed in the text.

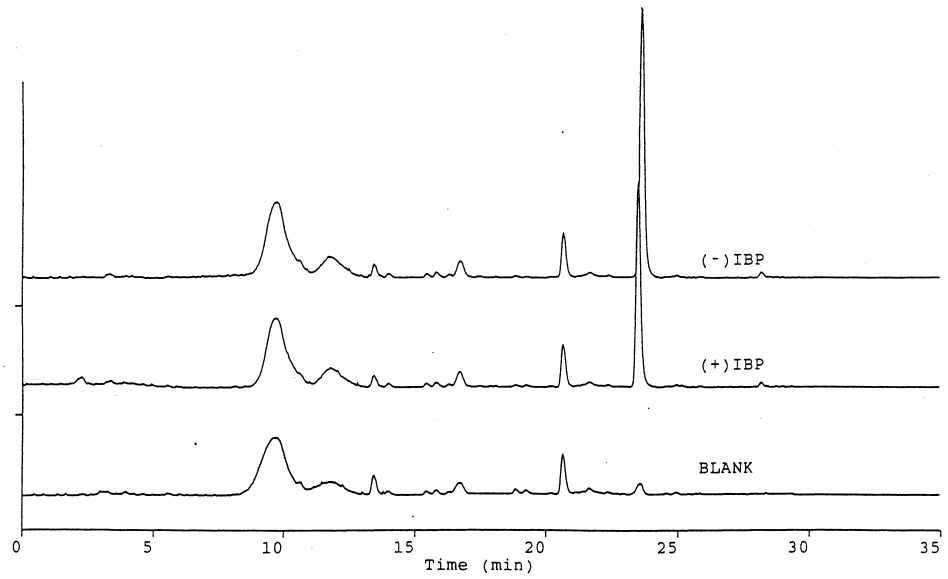

Fig. 5. Chromatograms of methylated dansyltyrosine derivatives of ibuprofen. HPLC conditions were the same as those listed in the text.

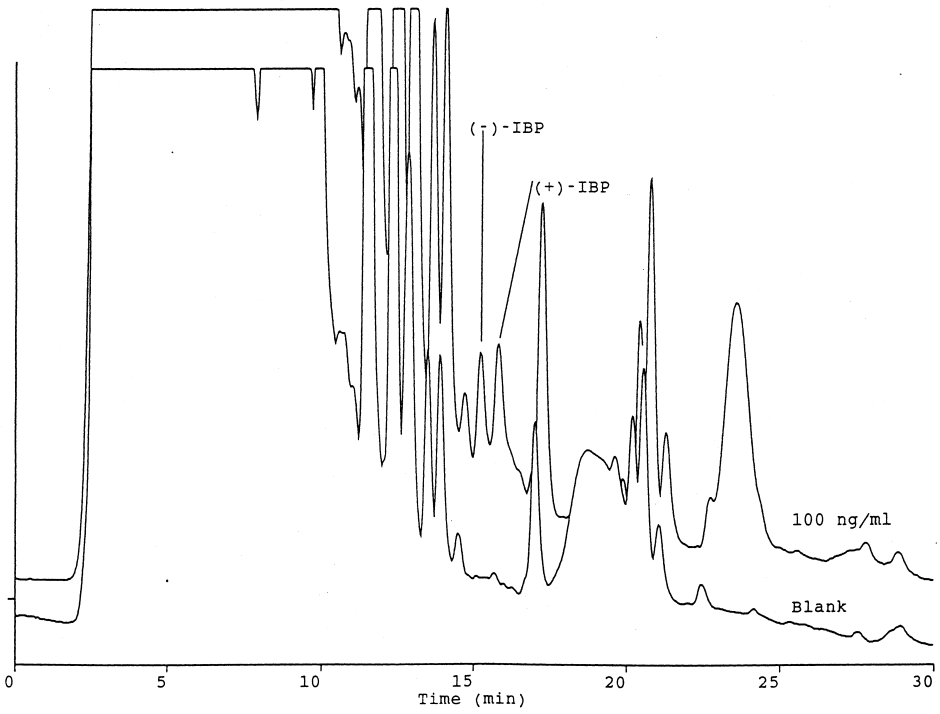

Fig. 6. Chromatograms of methylated dansyllysine derivatives of ibuprofen extracted from spiked human urine. HPLC conditions were the same as those listed in the text.

5 min under mild conditions. We established that other carboxylic acids, such as fatty acids, also reacted with the reagent under the same conditions (data not shown). Chromatographic resolution can be achieved on a conventional reversed-phase column. In contrast, the reported reagents described above require long derivatisation times and enantiomeric resolution can be achieved on normal-phase columns, which are not the columns of choice for biological samples. Thus, methylated Dns-Lys was proved to be a potent and facile fluorogenic reagent and to have a function contributing to enantiomeric separation on a conventional reversed-phase column.

4. Conclusion

We have developed a new labeling reagent, methylated dansyllysine, for compounds that have carboxylic moieties. It was demonstrated that the reagent readily reacted under mild conditions with few adverse reactions. It enabled sensitive determinations due to its strong fluorescence and it provided the enantiomeric resolution of chiral carboxylic compounds on a conventional reversed-phase column. These favorable characteristics are likely to facilitate the study of dynamic changes of both chiral and achiral carboxylic compounds. Moreover, as the reagent was shown to be useful for the quantitation of the drug compound in urine, it can also be used to study the pharmacokinetics of drug compounds.

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