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Reversed-phase high-performance liquid chromatographic separation of the enantiomers of trimetoquinol hydrochloride by derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and application to the optical purity testing of drugs

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ABSTRACT

The enantiomers of the bronchodilator trimetoquinol hydrochloride were separated by reversed-phase high-performance liquid chromatography after derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) reagent. The corresponding diastereomeric thiourea derivatives were baseline resolved on an ODS column with a resolution of more than 2 within 10 min. The derivatization proceeded smoothly and quantitatively within 15 min at room temperature even in aqueous solution. The favourable UV adsorption of the derivatized enantiomers permitted the detection of the (R)-$(+)$-isomer in (S)-$(-)$-trimetoquinol hydrochloride down to the 0.2% level. The determination of the optical purity of trimetoquinol hydrochloride drug substance and in tablets and injections was succesfully achieved. The optimization of the derivatization procedures and separation conditions is described.

INTRODUCTION

The enantiomers of different drugs, which have one or multiple asymmetric centres, may differ widely in their biological activities and toxicological properties [1,2] and it is therefore important to establish methods for the enantiomeric purity testing of chiral drugs. Trimetoquinol hydrochloride [(S)-$(-)$-form], which was developed at Tanabe Seiyaku as a broncodilator for use in bronchospastic diseases, such as asthma, has been found to be therapeutically more effective than the (R)-$(+)$-isomer, which has weak cardiovascular and bronchodilator activity [3]. A method for the determination of the optical purity of trimetoquinol hydrochloride and that in preparations such as tablets and injections is required in order to certify the quality. It is also required for the method to be simple, sensitive and reproducible.

Chromatographic methods have been widely used to separate and quantify the enantiomers of compounds in mixtures, especially high-performance liquid chromatography (HPLC). Recently much work has been conducted to separate enantiomers

directly using chiral stationary phases [4,5]. Numerous theoretical studies on chiral recognition have also been conducted to establish the mechanism of the separation of enantiomers and to select suitable chiral stationary phases according to their mode of separation mechanism and the type of chiral compounds, which have different functional groups and structures [4–6]. Although several commercially available chiral stationary phases have been used to achieve the successful separation of the enantiomers of the drug, it was unsuccessful for trimetoquinol hydrochloride. The chiral derivatization method was then employed to separate the enantiomers.

(S)-(−)-N-1-(2-Naphthylsulphonyl)-2-pyrrolidine carbonyl chloride (NSP-Cl), which can react with a hydroxy or an amino group, and which has been successfully applied to the separation of the enantiomers of diltiazem hydrochloride [7] and DL-amino acids [8], was first employed as a chiral reagent. Separation of the corresponding diastereomers of trimetoquinol with NPS-Cl was achieved by normal-phase HPLC and has been reported elsewhere [9]. The derivatization with NSP-Cl proceeds in non-aqueous media and with a small amount of alkali. This is a useful method for determining the optical purity of drug substances. However, it requires evaporation of the large volume of water used for the derivatization of the drug in injections, where the drug substance is dissolved in an aqueous solution. Sample treatment is very tedious and time consuming for routine analysis.

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT or GITC), which reacts with amino compounds even in aqueous media and has been successfully applied to the chiral separation of DL-amino acids [10,11] and β-blocking agents [12], was subsequently employed. Trimetoquinol reacted readily with TAGIT at room temperature and with a small amount of alkali in aqueous media. The corresponding diastereomers were successfully separated by an analytical ODS column within 10 min. We have been investigating the chiral separation or chiral recognition of drugs by reversed-phase HPLC. This mode is more useful than normal-phase HPLC for the determination of enantiomeric pairs of drugs in biological or aqueous solutions. Reversed-phase HPLC is also more reproducible than normal-phase HPLC, which is sensitive to moisture in the mobile phase.

This paper describes the reversed-phase HPLC chiral separation of the enantiomers of trimetoquinol hydrochloride. The optimization of the derivatization procedures and HPLC conditions is discussed. Applications to the determination of the optical purity of the drug substance and in tablets and injections are also described. Simple and rapid optical purity testing of trimetoquinol hydrochloride was accomplished.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of an LC-3A high-pressure pump, a CT-O column oven and an SPD-2A variable-wavelength UV detector (Shimadzu, Kyoto, Japan). A Shimadzu SPD-6MA photodiode-array detector was also used to monitor the UV spectra of peaks. Samples were applied to the column with a Rheodyne 7125 loop injector. An ODS column (150 mm × 4.6 mm I.D.) was used, slurry packed with Inertsil ODS-2 (particle size 5 μm) (Gasukuro Kogyo, Tokyo, Japan). Peak integration was carried out with a Chromotopac C-R5A dataprocessor (Shimadzu). Quanti-

fication of the (+)-antipode of trimetoquinol hydrochloride was effected using the equation

$$(+)\text{-antipode (\%)} = \frac{\text{peak area of } (+)\text{-antipode}}{\text{sum of peak areas of } (+)\text{-antipode and } (-)\text{-trimetoquinol}} \cdot 100$$

Materials

The chiral reagent TAGIT was purchased from Wako (Tokyo, Japan). Trimetoquinol hydrochloride [(S)-(−)-form], its racemate and Inolin tablets (3 mg of trimetoquinol per tablet), Inolin injections (0.1 mg in 1 ml and 0.05 mg in 20 ml) were obtained from the research laboratories at Tanabe Seiyaku (Osaka, Japan). The structure of (S)-(−)-trimetoquinol hydrochloride is shown in Fig. 1. HPLC-grade acetonitrile and methanol, and analytical-reagent grade N,N-dimethylformamide (DMF) were purchased frm Katayama Kagaku Kogyo (Osaka, Japan). Water was purified with a Millipore RO-60 water system (Millipore Japan, Tokyo, Japan). All other reagents and solvents were of analytical-reagent grade from Katayama Kagaku Kogyo. All quantitative volume transfer work at the microlitre level was performed with an Eppendorf digital pipetter (VWR, IL, U.S.A.) and a 10-μl syringe (Hamilton, NV, U.S.A.).

Chromatographic conditions

Optical resolution was carried out with the Inertsil ODS-2 column at a flow-rate of 1.0 ml/min and 40°C. The derivatized chiral drug was monitored with a UV detector at 250 nm, which is the wavelength of maximum absorption of the TAGIT reagent. The UV spectra of each derivatized enantiomer were also measured with a photo-diode-array detector over the wavelength range 200–300 nm.

The mobile phase consisted of 0.05 M phosphate buffer (pH 3.0)–acetonitrile (29:21, v/v). The buffer was prepared by dissolving 6.8 g of potassium dihydrogen-phosphate in 1000 ml of water, and the pH was adjusted to 3.0 with diluted phosphoric acid (*ca.* 10%). The mobile phases were passed through a membrane filter of 0.45-μm pore rize and 4.7-cm diameter (Fuji Photo Film, Tokyo, Japan) and were degassed by using a Branson Model B-2200 ultrasonic cleaner (Yamato, Tokyo, Japan) prior to use.

Derivatization of drug substances

About 1 mg of trimetoquinol hydrochloride or its racemate was dissolved in 1 ml of DMF. To 100 μl of this solution were added 100 μl of 0.5% (w/v) TAGIT solution in

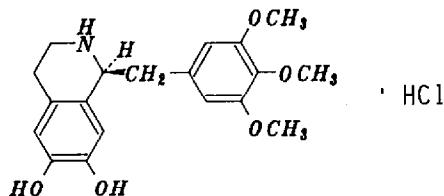
Fig. 1. Structure of (S)-(−)-trimetoquinol hydrochloride.
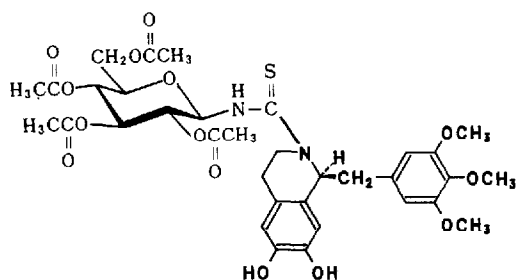
Fig. 2. Structure of TAGIT-derivatized (*S*)-(−)-trimetoquinol.

acetonitrile and 2 μl of triethylamine. The mixture was shaken vigorously and allowed to stand at room temperature for 15 min. The corresponding thiourea derivative was formed in this step and has the structural formula as shown in Fig. 2. After reaction, 2 μl of ethanolamine were added and the solution was shaken well and allowed to stand for 5 min to remove the excess of TAGIT reagent. Finally, 200 μl of 10% acetic acid were added and this acidic solution was used as a sample solution.

Derivatization of drug substances in tablets

Inolin tablets, each of which contains 3 mg of trimetoquinol hydrocloride, were extracted by DMF as follows. Twenty tablets were weighed accurately and ground, the powder, containing nearly 10 mg of trimetoquinol hydrochloride, was transferred into 10-ml volumetric flask and 7 ml of DMF were added. The flask was then sonicated for 10 min and warmed in a water bath (*ca*. 40°C) for 10 min with occasional shaking and cooled. After dilution to volume with DMF, the flask was shaken well, the solution was filtered and 100 μl of the filtrate were derivatized using the same procedure as described above.

Derivatization of drug substances in injections

Inolin injections contain 0.1 mg of trimetoquinol hydrochloride per ampoule (ampoule volume 1 ml) and those for intravenous injection contain 0.05 mg per ampoule (ampoule volume 20 ml). An ampoule of Inolin injection (0.1 mg) was cut and 200 μl of the solution were pipetted into a 2-ml vial and 200 μl of 0.5% (w/v) TAGIT solution in acetonitrile and 10 μl of 3% ammonia solution were added. The vial was shaken and allowed to stand at room temperature for 15 min. After reaction, the solution was subjected to the same procedure as for drug substances.

To 1 ml of Inolin intravenous injection were added 200 μl of 0.5% (w/v) TAGIT solution in acetonitrile and 10 μl of 3% ammonia solution, and the solution was subjected to the same procedure as for Inolin injection (0.1 mg), except for the addition of 10% acetic acid (100 μl).

Aliquots (10–20 μl) of these sample solutions were then injected into the HPLC system using a 25-μl microsyringe (Hamilton) and quantification of the antipode was effected by calculation from the peak areas of each enantiomer according to the above equation.

Preparation of blank

A blank for the derivatization of the enantiomeric drug was prepared by using the same procedure in the absence of drug substance.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Separation of the derivatized racemate of the chiral drug was investigated with the analytical ODS column (150 mm × 4.6 mm I.D.), using a mobile phase consisting of 0.05 M phosphate buffer (pH 3.0) and an organic solvent. Capacity factors (k'), separation factors (α) and resolution (R_s) of corresponding diastereomers obtained with various concentrations and types of organic solvent are summarized in Table I. The TAGIT-derivatized trimetoquinol racemate was baseline resolved by reversed-phase HPLC using acetonitrile or methanol as the organic solvent with a short analysis time. Methanol was more effective than acetonitrile for the resolution of the diastereomers, as judged from the resolution values and analysis times, although baseline resolution was achieved with both. With the use of methanol in the mobile phase, unreacted TAGIT reagent eluted near the peak of (S)-$(-)$-trimetoquinol and the column pressure drop was higher than with the acetonitrile-containing mobile phases. Acetonitrile was therefore selected as the organic modifier of the mobile phase. In the separation of TAGIT-derivatized DL-amino acids, acetonitrile was effective for the resolution of TAGIT–DL-serine, which was not resolved using methanol. On the contrary, methanol was effective for the resolution of TAGIT–DL-arginine, which was not baseline resolved with the acetonitrile-containing mobile phase. Thus, the simultaneous separation of common protein amino acids enantiomers was successfully achieved by gradient elution using a ternary mobile phase of methanol, acetonitrile and buffer solution [11].

A typical chromatogram of TAGIT-derivatized trimetoquinol racemate in a mobile phase of acetonitrile–phosphate buffer (pH 3.0) (21:29) is shown in Fig. 3. UV spectra of each peak monitored by using a Shimadzu SPD-6MA photodiode-array detector are shown in Fig. 4. Each diastereomer shows an identical spectrum of the

TABLE I

CAPACITY FACTORS, SEPARATION FACTORS AND RESOLUTION OF TRIMETOQUINOL RACEMATE USING METHANOL AND ACETONITRILE AS AN ORGANIC MODIFIER

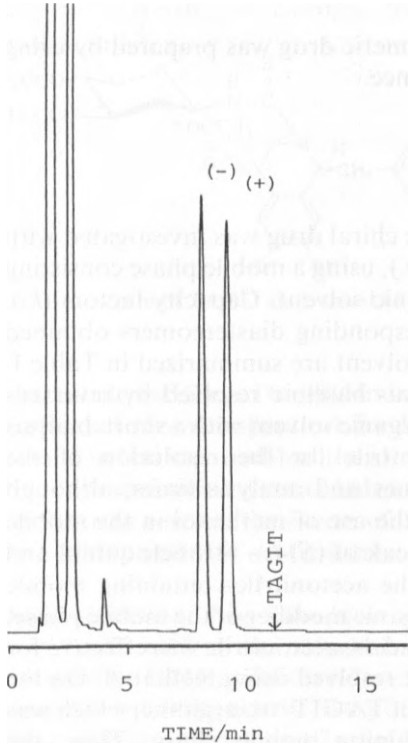| Organic modifier	Content (%)	k'		α	R_s
		$(-)$-	$(+)$-		
Methanol	60	1.67	2.29	1.37	2.97
	57.5	2.27	3.12	1.37	3.42
	56	3.15	4.33	1.37	3.74
	55	4.14	5.66	1.37	4.14
Acetonitrile	44	2.63	3.03	1.15	1.79
	42.5	2.94	3.41	1.16	1.92
	42	3.49	4.06	1.16	2.10
	41	3.89	4.54	1.17	2.27

Fig. 3. Separation of the trimetoquinol racemate after derivatization with TAGIT. Mobile phase, 0.05 *M* phosphate buffer (pH 3.0)–acetonitrile (29:21), Column, Inertsil ODS-2 (150 mm × 4.6 mm I.D.); temperature, 40°C. Flow-rate, 1.0 ml/min. Detection, 250 nm.
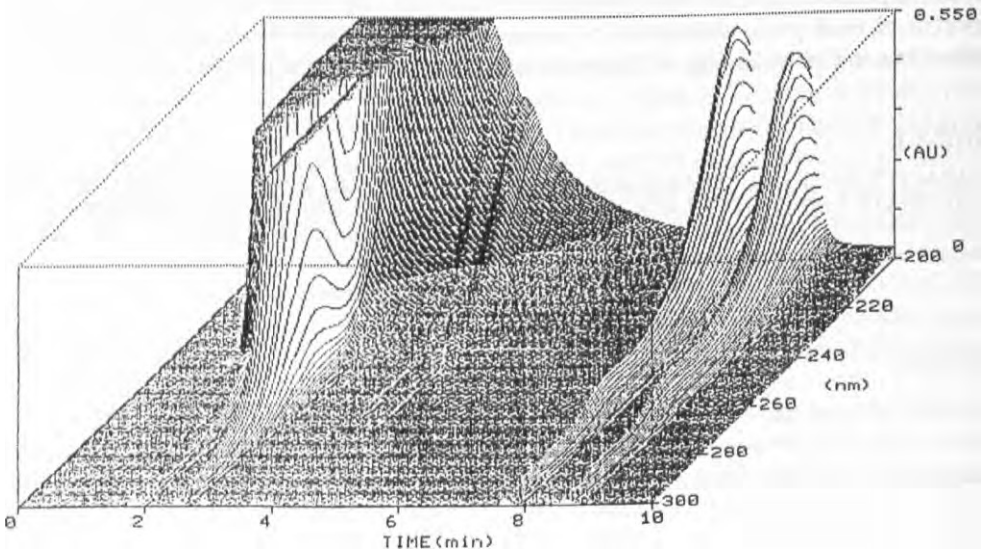

Fig. 4. UV spectra of each diastereomer monitored by a photodiode-array detector.

maximum absorption at 208 nm. Trimetoquinol hydrocloride itself shows the maximum absorption at 283 nm in 0.01 M hydrochloric acid solution [13]. Separation was successfully achieved within 10 min without interference from the TAGIT peak (see Fig. 3) or reagent blank peaks (see Figs. 5 and 6). Trimetoquinol [(S)-(−)-form] eluted faster than the antipode [(R)-(+)-form]. This elution order (S)-(−)-isomer > (R)-(+)-isomer corresponds to the results for other TAGIT derivatized drugs such as β-blocking agents [12] and antihypertensive agents [14]. However, for denopamine, which is a cardiotonic agent, the elution order was (R)-(−)-isomer > (S)-(+)-isomer [15]. The excellent resolution is probably due to the lipophilic nature of the tetraacetylglucopyranosyl residue of TAGIT and its conformational rigidity [10,11].

Optimization of derivatization conditions

Optimization of the derivatization of trimetoquinol hydrochloride with TAGIT was investigated by varying the reaction temperature (room temperature and 40°C), reaction time (from 5 to 60 min) and the amounts of TAGIT reagent (two, five and ten times that of of the drug substance) and alkali. The effect of the species of alkali on the reactivity was also investigated. The derivatization conditions were selected so as to result in the greatest peak height of the TAGIT-derivatized trimetoquinol. The derivatization proceeded rapidly within 15 min without warming with an excess of TAGIT (more than five times that of the sample) and a small amount of alkali. Concerning the type of alkali, reaction of the drug substance and those in tablets readily proceeded by using triethylamine, as in the derivatization of amino acids. However, for Inolin injections (aqueous solutions), dilute ammonia solution (*ca.* 3%) was more effective for the reactivity than triethylamine and a 1 M sodium hydroxide solution and its use gave the maximum peak height of the derivative, although ammonia itself can react with TAGIT. The use of dilute ammonia solution to catalyse the reaction was not a problem because unreacted TAGIT remained in sufficient amount even after reaction for 15 min, as judged from its peak area. The peak area was, however, reduced by half after 45 min in comparison with that after a 15-min reaction. The reaction blank in the chromatogram obtained with the use of ammonia solution (*ca.* 3%) was also smaller than that with other alkalis.

The peak areas of TAGIT-derivatized trimetoquinol hydrochloride in injections (0.1 mg) obtained with various reaction times at room temperature are summarized in Table II together with the data on the stability of the derivative in the reaction solution. With an excess of TAGIT reagent, the reaction proceeded rapidly and quantitatively, as judged from the constant peak areas of the derivatives for reaction times from 10 to 60 min. The peak obtained from a reaction mixture that was warmed at 40°C in a water-bath for 15 min had the same area as that obtained at room temperature.

The conditions selected are described under Experimental.

Linearity of response and limit of detection

Linearity of response for trimetoquinol hydrochloride [(S)-(−)-form] was investigated by derivatizing samples with a concentration range 20–120% of the concentration of drug substance in the sample (0.1%, w/v). The graph passed through the origin and the peak areas exhibited linearity over the tested range with a correlation coefficient $r = 0.996$.

The recovery of peak-area response of the (R)-(+)-isomer added to (S)-(−)-

TABLE II

PEAK AREA OF TAGIT-DERIVATIZED (S)-(−)-TRIMETOQUINOL AS A FUNCTION OF REACTION TIME AND STABILITY OF THE SAMPLE SOLUTION AT ROOM TEMPERATURE

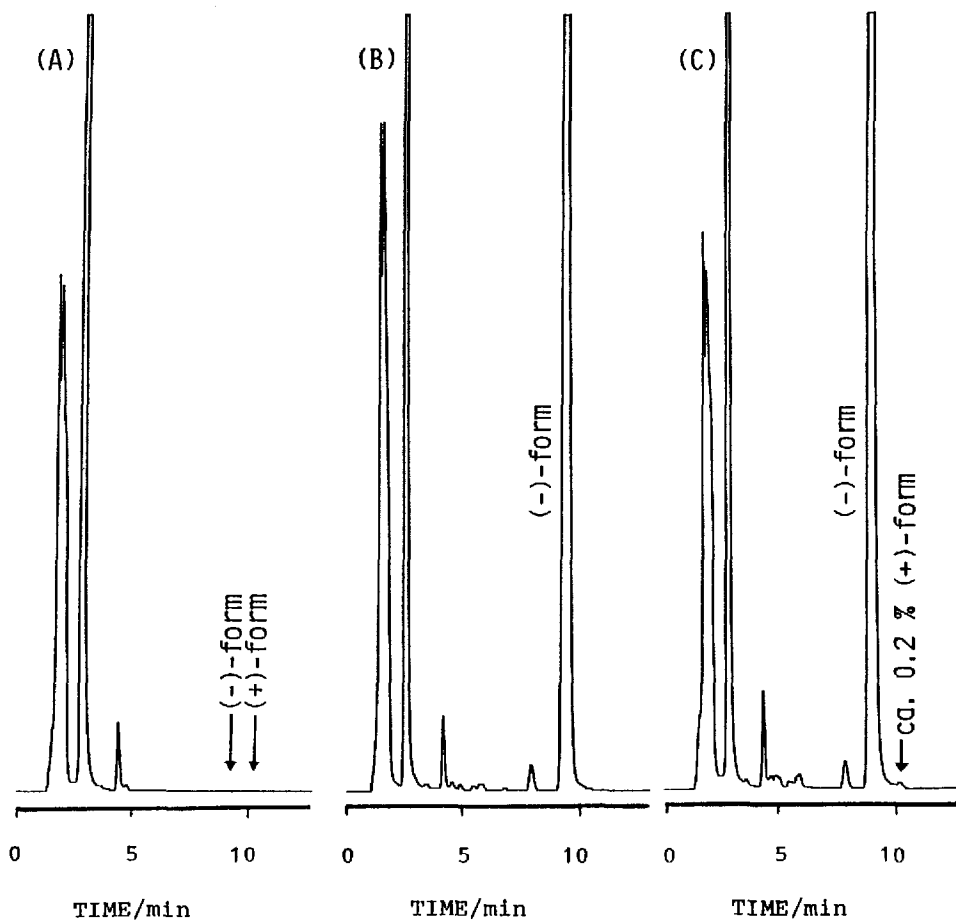|	Reaction time (min)				Storage period (days)	
	5	10	15	60	1	4
Peak area (μV s $\times$ 10^4)^a	48.2	50.9	50.4	49.9	49.1	49.4

^{a} Average value ($n = 3$).

Fig. 5. Chromatograms of (A) a blank, (B) standard (S)-(−)-trimetoquinol and (C) standard trimetoquinol with *ca.* 0.2% of (+)-isomer added. Conditions in Fig. 3.

trimetoquinol hydrochloride was also examined over the range 0.2–2.0% (w/w) added. The recovery of the antipode averaged 98.2% over this addition range. The graph also passed through the origin with a slope of 1.07, an intercept of 0.03 and a correlation coefficient $r = 0.994$.

These results indicate that the extent of derivatization reaction of the (R)-$(+)$- and (S)-$(-)$-isomers with TAGIT is independent of the amount of each enantiomer presented in the reaction mixture or whether the reactivity of each enantiomer with TAGIT is the same. That is, the peak-area ratio of the derivatized product is equal to the weight ratio of the underivatized $(+)$- and $(-)$-isomers. Chromatograms of optically pure (S)-$(-)$-trimetoquinol hydrochloride, (S)-$(-)$-trimetoquinol with *ca.* 0.2% of the (R)-$(+)$-antipode added and a blank are shown in Fig. 5. It was found that the limit of detection of the $(+)$-isomer under these conditions was down to the 0.2% level.

Stabilities of sample and reagent solutions

The excess of TAGIT reagent that remains after the derivatization of the drug was removed by addition of ethanolamine, resulting in a faster elution than that of the TAGIT-derivatized drugs. The peak of TAGIT-derivatized ethanolamine appeared at *ca.* 2.5 min (see Figs. 3 and 4). Finally, the solution was acidified to *ca.* pH 3.0 by the addition of 10% acetic acid as described above. The acidic sample solutions were stable for at least 4 days at room temperature, as judged from the constant peak areas of the TAGIT-derivatized drug shown in Table II. This will allow overnight analysis by using an auto-sampler in the routine optical purity testing of many samples. The reactivity of TAGIT reagent in acetonitrile also did not decrease for at least 1 week.

Optical purity testing of trimetoquinol hydrochloride and those in preparations

Determination of the optical purity of drug substances and those in tablets was performed according to the described procedure. Trimetoquinol hydrochloride was extracted from the tablet matrix quantitatively by using DMF with sonication and warming, owing to its good solubility in DMF. The (R)-$(+)$-isomer was not detected in all batches of drug substances and Inolin tablets tested. These have excellent optical purity of more than 99.8%, as shown in Table III.

TABLE III

RESULTS OF OPTICAL PURITY TESTING OF TRIMETOQUINOL HYDROCHLORIDE AND THAT IN PREPARATIONS

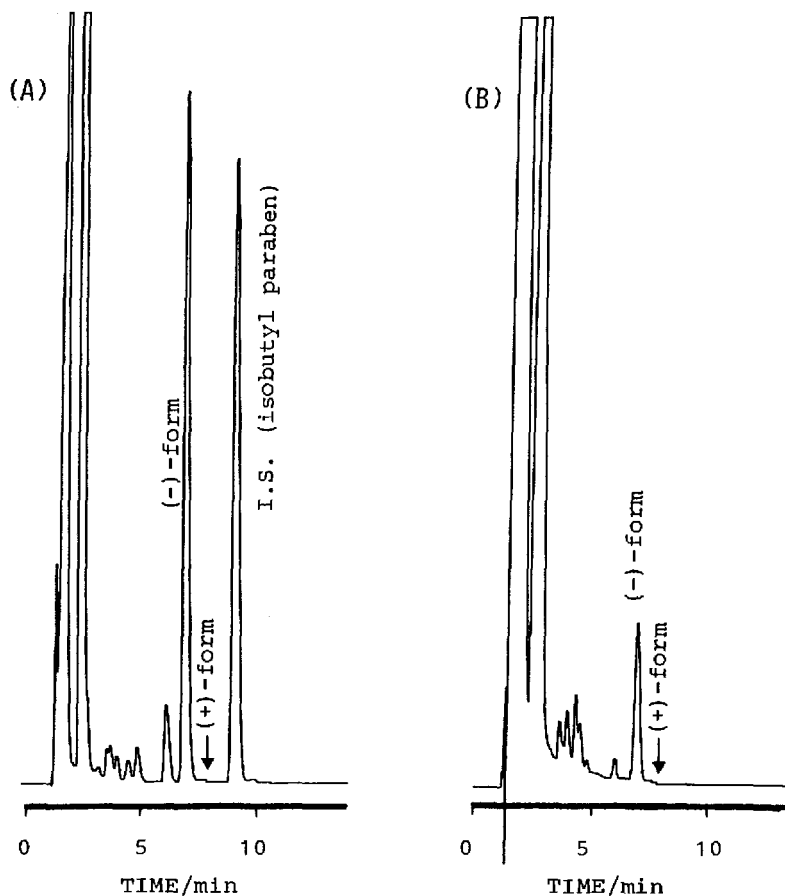| Sample	Batch	Optical purity (%)
Trimetoquinol hydrochloride	AQ-1	>99.8
	AQ-2	>99.8
Inolin tablets (3 mg)	IJ-1	>99.8
	IJ-2	>99.8
Inolin injection (0.1 mg)	IC-1	>99
Inolin injection (0.05 mg)	IC-2	>95

Fig. 6. Chromatograms for (A) the assay and optical purity testing of Inolin injection (0.1 mg) and (B) optical purity testing of Inolin intravenous injection. Conditions in Fig. 3 except that the flow-rate was 1.1 ml/min.

Assay and optical purity testing of Inolin injections (0.1 mg) were successfully performed by using p-hydroxyisobutyl benzoate as an internal standard (I.S.). A typical chromatogram is shown in Fig. 6A. An I.S. solution was prepared by dissolving the substance in 10% acetic acid, the solution obtained being added to the reaction mixture to acidify the solution in the last step of the derivatization (100 or 200 μl) to give a peak-area ratio of the drug to I.S. of ca. 1. A standard solution for the assay was prepared by dissolving a known amount of the drug and adding the I.S. solution using the same procedure as for the sample solution preparation. The averaged assay value for ten ampoules of Inolin injection (0.1 mg) was 103.6% with a relative standard deviation of 2.9%. The (R)-($+$)-isomer was not detected (Table III). Both assay and optical purity testing could be performed simultaneously in one analysis. The limit of detection for this preparation was almost 1%. The optical purity of the Inolin injections (0.1 mg) was more than 99% in all instances. The optical purity of Inolin

intravenous injections could also be successfully determined by the same method, as shown in Fig. 6B, although the limit of detection was about 5%.

CONCLUSIONS

The reversed-phase HPLC method presented here is simple, relatively sensitive and reproducible. The derivatization of trimetoquinol hydrochloride with TAGIT proceeded rapidly and quantitatively at room temperature. The derivatized drug is stable in acidic solution and the TAGIT reagent solution. Baseline resolution of the diastereomers was achieved within 10 min on an analytical ODS column. Optical purity testing of the drug substance and in tablets and injections was also successfully achieved. This method may be useful for separating the enantiomers of other drugs or compounds which have functional groups that react with TAGIT, with minor alterations to the chromatographic and derivatization conditions.

ACKNOWLEDGEMENTS

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