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DETERMINATION OF ENANTIOMERIC PURITY OF THE NEW D-2 DOPAMINE AGONIST 2-(N-PROPYL-N-2-THIENYLETHYLAMINO)-5-HYDROXYTETRALIN (N-0437) BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER PRE-COLUMN DERIVATIZATION WITH D(+)-GLUCURONIC ACID

T.K. GERDING*, B.F H. DRENTH, V.J.M. VAN DE GRAMPEL, N R. NIEMEIJER and R.A. DE ZEEUW

Department of Toxicology, State University, A. Deusinglaan 2, 9713 AW Groningen (The Netherlands)

and

P.G. TEPPER and A S. HORN

Department of Medicinal Chemistry, State University, A. Deusinglaan 2, 9713 AW Groningen (The Netherlands)

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SUMMARY

This paper describes an enzymic derivatization procedure that allows accurate determination of very small amounts of enantiomeric impurities in the D-2 dopamine agonist 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin (N-0437). After pre-column glucuronidation of the individual enantiomers, two diastereoisomers were formed which were separated by reversed-phase high-performance liquid chromatography. An enantiomeric purity of 99.84% was calculated for the (−)-enantiomer, against 99.89% for the (+)-enantiomer. The assay was validated by spiking 1% of the (−)-enantiomer in the (+)-enantiomer. A high accuracy (error 4.5%) and precision (coefficient of variation 2.9%, $n=5$) of the method were established.

INTRODUCTION

Over the past few years, numerous articles have reported that enantiomers can possess different biological activity and toxicity. Moreover, differences in absorption, distribution, metabolism and excretion between the individual enantiomers may exist [1,2], so individual enantiomers as well as the racemic drug must be evaluated.

In our institute the dopamine agonist 2-(N-propyl-N-2-thienylethylamino)-5-

hydroxytetralin (N-0437)[a] was developed [3]. It was tested as a racemate in vivo and in vitro and shown to be very potent and selective for the D-2 receptor [4,5]. A recent study with the enantiomers revealed that III is selective for presynaptic receptors, implicating a potential therapeutic use against schizophrenia, while II preferentially stimulates postsynaptic dopamine receptors, thereby being applicable against Parkinson's disease [6]. It was established that the levo-isomer has an affinity for the D-2 receptor 140 times higher than that of the dextro-isomer [7]. In this study nuclear magnetic resonance (NMR) was used to establish the enantiomeric purity, but, in order to interpret the results of pharmacological experiments correctly, especially when large differences in pharmacological activity exist, it is extremely important to determine the enantiomeric purity accurately. A variety of methods is available [18], but only a few techniques are capable of providing a reliable value of the enantiomeric purity in the range between 98 and 100%.

Determination of the rotation is not suitable as the precision of polarimetric measurements rarely is better than 2%. Besides this, the presence of other impurities constitutes an additional error.

In our case the measurement of the optical rotation of the separate enantiomers provided no direct information, as no true value of the specific rotation was known. Also NMR, isotope dilution and differential microcalorimetry are inadequate for the determination of trace amounts of enantiomeric impurities. Only gas chromatography (GC) and high-performance liquid chromatography (HPLC) have proven to be convenient methods to establish enantiomeric compositions accurately [9–12], performed in either the direct or the indirect mode. In a recent note we reported the separation of the enantiomers of I by reversed-phase (RP) HPLC after pre-column derivatization with D(+)-glucuronic acid [13]. By coupling D(+)-glucuronic acid to the phenolic group of I (see Fig. 1), catalysed by the enzyme uridine 5'-diphosphoglucuronyltransferase (UDPGT), two diastereoisomeric glucuronides were formed, which could be separated by a non-chiral chromatographic system.

Of course, a derivatization method can be utilized only if the reaction rates of both enantiomers with the optically active reagent are similar, or a quantitative
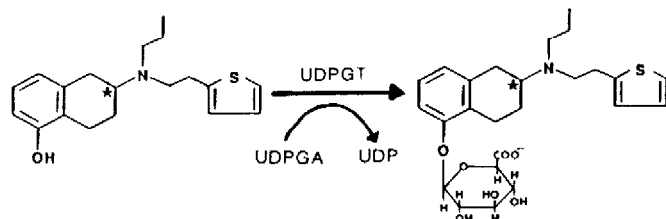

Fig. 1. Glucuronidation of 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin catalysed by the enzyme uridine 5'-diphosphoglucuronyltransferase (UDPGT), using the co-substrate uridine 5'-diphosphoglucuronic acid (UDPGA). The star denotes the position of the asymmetric carbon atom.

[a] In the following text racemic N-0437 will be indicated by I, the (−)-enantiomer, known as N-0923, by II and the (+)-enantiomer, named N-0924, by III.

derivatization within an acceptable reaction time is achieved. Especially when an enzymatic derivatization method is employed, one must avoid changes in the original enantiomeric ratio due to different reaction rates and/or equilibrium constants of the enantiomers. Also, the glucuronidation reaction has been reported to be stereoselective [14–17]. Therefore, it was necessary to ensure that quantitative glucuronidation of both enantiomers occurred within the incubation time by determining the enzyme kinetic parameters (V_{max} and K_M) as well as the derivatization yield.

EXPERIMENTAL

Chemicals

The drug I was synthesized by Horn et al. [3] and was found to be more than 99% pure, as determined by HPLC and thin-layer chromatography (TLC). The enantiomers were obtained by crystallization of the precursor 5-methoxy-N-propyl-2-aminotetralin with 4-(2-chlorophenyl)-5,5-dimethyl-2-hydroxy-1,3,2-dioxaphosphorinane 2-oxide [18], followed by a separate conversion of the (+)- and (−)-forms into the (+)-and (−)-enantiomers of I. The compounds I, II and III were isolated and used as their hydrochlorides.

Tritium-labeled I was obtained from Amersham (Buckinghamshire, U.K.) with a specific activity of 87.4 Ci/mmol. The radiochemical purity was checked by TLC and HPLC and found to be 97%. Uridine 5'-diphosphoglucuronic acid (UDPGA) and 1-octanesulphonic acid (sodium salt) were purchased from Sigma (St. Louis, MO, U.S.A.). Water was obtained from a Milli-Q water purifier (Millipore, Bedford, MA, U.S.A.). All other chemicals used were of analytical grade and obtained from Merck (Darmstadt, F.R.G.).

Incubation procedures

Fresh bovine livers were obtained from a slaughterhouse (Eelde, The Netherlands). Bovine microsomes were prepared according to the procedures described previously [19].

The microsomal pellet was suspended in 125 mM Tris–HCl (pH 7.4) to a protein concentration of 10.2 mg/ml, determined according to Lowry et al. [20], and stored at −80°C. Incubations were performed in polyethylene cups (Eppendorf®, ca. 1.5 ml). The medium consisted of 80 mM Tris–HCl buffer (pH 7.4), 50 mM magnesium chloride, 6 mM UDPGA, 300 μM substrate (I, II and III) and 2 mg of microsomal protein in a final volume of 1 ml.

The enzyme for the glucuronidation, UDPGT, was activated by Mg^{2+} [21] during a preincubation period of 10 min at 37°C in a water-bath, with constant shaking. The addition of 0.01% Triton X-100, used previously [13], proved to be superfluous as no decrease in the amount glucuronidated was observed when the Triton was omitted, established according to the method described under derivatization yield. After 5 min of preincubation, the substrates were added. The reactions were initiated by the addition of the co-substrate UDPGA and, after vortex-mixing for 5 s, the mixtures were incubated at 37°C for 120 min with constant shaking. The reactions were terminated by placing the vials on ice. After

centrifugation of the incubation mixtures at 8000 g for 10 min in a Heraeus Christ Biofuge A (Osterode am Harz, F.R.G.) a microsomal pellet was obtained. The clear supernatant was decanted and stored at $-20°$C until analysis by HPLC.

HPLC conditions

The supernatants from the incubation mixtures were analysed by a system consisting of a Spectra-Physics (SP) 8770 pump (Santa Clara, CA, U.S.A.) coupled to a Shimadzu SPD-6A variable-wavelength UV detector, operating at 225 nm. The chromatograms were recorded on an Omniscribe® recorder (Houston Instruments, Austin, TX, U.S.A.).

Data were stored on an Apple computer provided with an Adalab® A/D converter (Interactive Microware, State College, PA, U.S.A.). Determinations were done by the Chromatochart® integration program (Interactive Microware). Injections were made using a Rheodyne 7125 injection valve (Cotati, CA, U.S.A.) fitted with a 50-μl sample loop. Separations were performed in a stainless-steel column (150 mm × 4.6 mm I.D.), packed with Rosil® C_{18} (particle size 3 μm), from Alltech (Zwijndrecht, The Netherlands). The mobile phase was 0.01 M phosphate buffer (pH 6.8)–acetonitrile (88:12). The chromatography was carried out at ambient temperature and a flow-rate of 1.5 ml/min.

Derivatization yields

In order to establish the derivatization yields of the glucuronidation reaction, four incubations were performed under exactly the same incubation procedures as mentioned above, using 300 μM I plus 10 μCi labeled I as substrates. The incubation mixtures were analysed by ion-pair chromatography (IPC), which is known to be a powerful technique for the separation of the parent compound and its corresponding glucuronidate [22, 23]. By complexation of a counter-anion with the protonated tertiary amine in I, an apolar neutral complex is formed. Thereby a selective retention of I and its glucuronide conjugates is achieved. The chromatographic system was chosen to establish a satisfactory separation of the conjugates from the solvent front within an acceptable total analysis time. A mobile phase of 0.01 M phosphate buffer (pH 2.5)–acetonitrile (68:32), containing 0.005 M octanesulphonate as counter-ion, and a flow-rate of 1.5 ml/min resulted in retention times of ca. 16 min and 4 min for I and its glucuronidate, respectively.

Aliquots of 50 μl of each sample were injected on the column (150 mm × 4.6 mm I.D.), packed with Nucleosil C_{18} (5 μm particles) (Macherey-Nagel, Düren, F.R.G.), according to Kuwata et al. [24]. The pH of the eluent was adjusted to 2.5 in order to effect total protonation of the tertiary amine and to suppress the ionization of the glucuronic acid group.

The column effluent was collected in fractions of 30 s into polyethylene Pony vials (Packard, Brussels, Belgium). After addition of 2 ml of RiaLuma (Lumac/3M, Schaesberg, The Netherlands), the radioactivity was measured in a B4450 Packard scintillation counter for 5 min or up to 40 000 counts. For quench correction the spectral index of the external standard was used.

The derivatization yield was calculated by dividing the amount of radioactivity

comprising the underivatized labeled I by the one representing the glucuronidate.

Reaction rate measurements

The glucuronidation rates of II and III were determined by incubating different concentrations (20–150 μM) under the same conditions as described under incubation procedures. As a high reaction rate is desirable, in order to effect a quantitative transformation within 120 min, the co-substrate UDPGA was added in at least twenty-fold excess over the substrate concentrations. The reactions were stopped after an incubation period of 10 min with 100 μl of 7% (v/v) perchloric acid. All reactions were performed under conditions that were linear with respect to protein concentration and time. The clear supernatants were analysed by IPC, as described above. The amount of glucuronide formed was calculated from the peak areas of the substrates. The corresponding concentrations of the parent compounds were established by extrapolation to standard curves of blank incubations, i.e. the same substrate concentrations incubated under identical conditions, without UDPGA. The enzyme kinetic parameters were calculated from a double reciprocal plot of the initial rate of conjugation as a function of substrate concentration (Lineweaver–Burk plot).

Assay validation

The assay was validated by spiking 1% (v/v) II in the stock solution of III, maintaining a total substrate concentration of 300 μM in the incubation mixture. The assay was carried out five times on different days, and the quantitation was done by peak integration. The enantiomeric purity was determined from the peak areas of the two diastereoisomers, assuming that no difference in molar absorptivities existed. This assumption is made reasonable by the fact that the ratio of the diastereomeric peak areas, after derivatization of I, is 1.02 [$(-)/(+)$ ratio, coefficient of variation (C.V.) 0.70%, $n=2$].

RESULTS

Fig. 2 shows the chromatograms after derivatization of 300 μM I (A), II (B) and III (C). For the glucuronide of II (II GA), a k' value of 45.5 was determined, against 51.3 for III GA. The resolution between the diastereoisomers was 3.60, and for the separations factor (α) a value of 1.13 was calculated. Increasing the percentage of acetonitrile to 16% resulted in a k' of 15.4 for II GA, against 16.8 for III GA, and a resolution of 2.42 ($\alpha=1.09$), while the total analysis was completed within 20 min. However, as we aimed to develop a method that provides an accurate determination of the enantiomeric purity in the range 98–100%, a higher priority was given to a high resolution than to a short analysis time. It must be noted that relatively large variations in retention times occurred when analyses were performed on different days in contrast to a low within-day variation: Fig. 2A was recorded on a different day from Fig. 2B and C, which were determined on the same day. On the basis of the peak areas, it was calculated that II contained 0.16% III (Fig. 2B), while III was contaminated with 0.11% of II
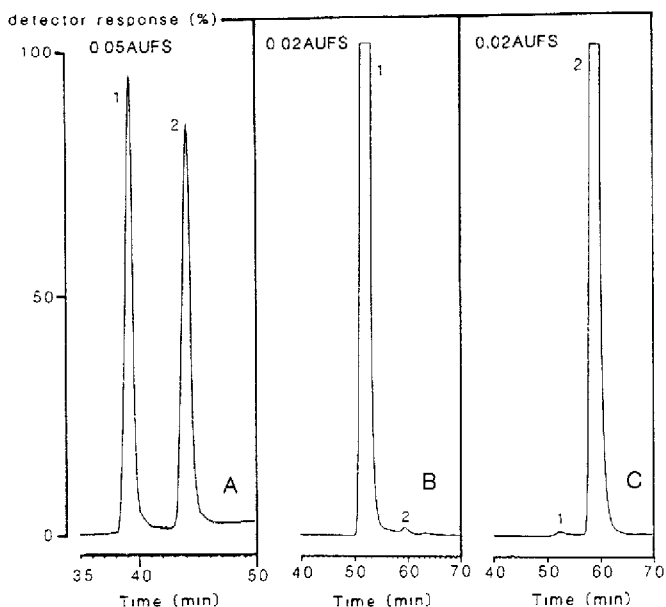
Fig. 2. Separation of the diastereomeric glucuronides of 2-(N-propyl-N-2-thienylethylamino)-5-hy-droxytetralin by RP-HPLC after pre-column derivatization of I (A), II (B) and III (C). Peaks: 1 = glucuronide of II; 2 = glucuronide of III. Chromatograms were recorded at 225 nm, after injection of 50 μl of supernatant. Stationary phase, Rosil® C_{18}; mobile phase, 0.01 M phosphate buffer (pH 6.8)-acetonitrile (88:12); flow-rate, 1.5 ml/min.
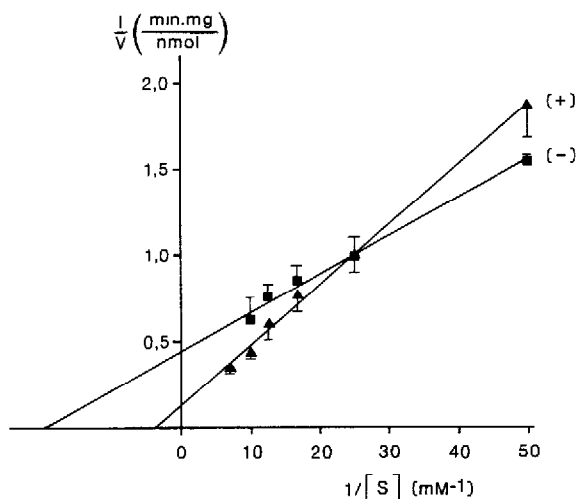

Fig. 3. Double reciprocal plot of the initial rate of glucuronidation as a function of the substrate (S) concentration. The UDPGT activity is expressed as nmol glucuronide formed per min per mg protein. Mean values $\pm$ S.E.M are plotted ($n = 3$) (■) Glucuronide of II, (▲) glucuronide of III.

(Fig. 2C). The percentage of underivatized I was determined to be 0.72% (C.V. = 19.1%, $n = 4$).

Standard curves of II and III showed excellent linearity over the concentration range 20–150 μM ($r^2 > 0.999$). The curves could be described by the equations

TABLE I

IN VITRO GLUCURONIDATION OF II AND III

Each value represents the mean of three experiments ± S.E.M.

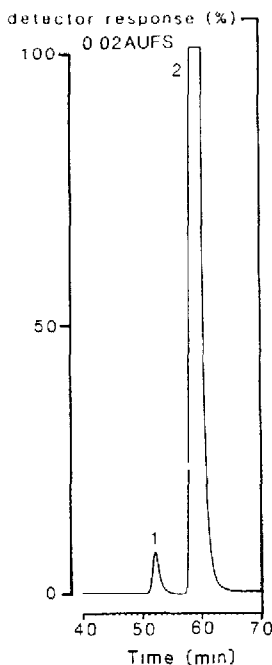| Substrate	V_{max} (nmol/min mg)	K_m (μM)	V_{max}/K_m (μl/min mg)
II	2.6 ± 0.9	58 ± 25	44 ± 3.7
III	7.1 ± 1.4	240 ± 45[a]	30 ± 3.0

[a]Statistically significant at $P < 0.05$ (Student's t-test).

Fig. 4. Separation of the diastereomeric glucuronides of III spiked with 1% of II. Peaks: 1 glucuronide of II; 2 glucuronide of III. Chromatographic conditions as described in Fig. 2.

$y = 606.1x + 2206$ and $y = 701.9x + 174.0$, for II and III, respectively, where y is the peak area and x is the enantiomer concentration (nmol/ml). The precision of the determination was very good, as established from the standard curve of II (the C.V. over the range 20–150 μM was less than 10%, $n = 3$).

The glucuronidation rates for both enantiomers were calculated from the Lineweaver–Burk plots, shown in Fig. 3. The enzyme kinetic parameters V_{max} and K_m are given in Table I. For the assay validation 1% (v/v) of the (−)-enantiomer was added to the stock solution of the (+)-enantiomer. The chromatogram after derivatization is presented in Fig. 4. A mean enantiomeric impurity of 1.07% was established (C.V. = 2.9%, $n = 5$). After correction for the intrinsic enantiomeric impurities present in the two batches (see Fig. 2), an error of 4.5% was calculated between the added and measured concentration.

DISCUSSION

In drug metabolism, the conjugation reaction with glucuronic acid is one of the major biotransformation routes in mammals [21]. The glucuronidation may take place with one or more functional groups, such as hydroxyl, carboxyl, amino, imino or sulphydryl groups. When the parent compound contains a chiral centre, the glucuronidation reaction results in the formation of two diastereoisomers. Using RP-HPLC, the separation of diastereomeric glucuronides has been described for oxazepam [25, 26], 2-arylpropionic acids [27] and propranolol [28]. After metabolic ring hydroxylation followed by glucuronidation, the separation of the diastereoisomers of 4′-hydroxypropranolol [28] and 5-(4-hydroxy-phenyl)-5-phenylhydantoin [29] has been reported. Considering the resolution of the diastereomers it seems that the separation is enhanced by the presence of bulky groups near the chiral centres or by a close proximity of the chiral centres in the conjugate. This is most clearly illustrated by comparison of the resolution of the propranolol diastereomers, which are derivatized at the hydroxyl directly attached to the chiral centre, with that of the 4′-hydroxypropranolol conjugates, in which the anomeric centre is separated by eight bonds from the chiral centre of the aglycone [28]. In the case of the glucuronides of I there are six bonds between the anomeric centre of the glucuronic acid and the aglycone, and close proximity between the chiral centres is further hindered by the rigidity of the aminotetralin ring. Therefore, a good separation between the two diastereomers could only be achieved in a relatively large analysis time.

The derivatization with glucuronic acid to determine the enantiomeric purity of the separate enantiomers offers several potential advantages:

(1) The derivatizing agent is present as optically pure D(+)-glucuronic acid, resulting in the formation of pure diastereoisomers.

(2) The reaction is cheap, easy to perform and requires mild reaction conditions, thereby avoiding thermal degradation or racemization.

(3) The indirect approach, i.e. the separation of pre-column-synthesized diastereoisomers, offers the advantage that a non-chiral HPLC system can be employed which is, through its high efficiency and separation power, most useful in the accurate determination of very small enantiomeric impurities.

For compound I, an excellent derivatization yield (more than 99% in 120 min) was established for the glucuronidation reaction. However, in order to determine the enantiomeric purity accurately, it must be ascertained that the enantiomeric ratio is not disturbed as a consequence of a high degree of stereoselectivity of the enzyme UDPGT. From the data listed in Table I, a $(+)/(-)$ enantiomeric ratio of 2.8 in V_{max} was calculated, while the affinity of the $(-)$-enantiomer for the enzyme was ca. four-fold higher. Assuming first-order conditions, a comparison between the glucuronidation rates of both enantiomers is best reflected by the V_{max}/K_m ratios [30], which are quite similar. Using this ratio, a difference of only 33%, in favour of the $(-)$-enantiomer, was established. Yet, even if we consider the initial glucuronidation rates (V_{max}) during the first 10 min to be representative for the total conjugation reaction, it can be calculated that both enantiomers are being converted quantitatively within the incubation time of 120 min (612

μM for II and 1704 μM for III, compared with a substrate concentration of 300 μM.

The assay validation showed a high accuracy and precision. Unfortunately, no further validation by spiking lower amounts of the $(-)$-enantiomer in the stock solution of the $(+)$-enantiomer and vice versa could be performed, owing to the lack of reference material.

It should be noted that towards the end of these studies, a degradation of the column was observed, resulting in a decreased separation of the diastereomers. As this degradation may be caused by soluble proteins present in the supernatants, the introduction of a deproteinization step could perhaps prevent this phenomenon. Further investigations are being carried out to evaluate which other drug structures can be analysed for their enantiomeric purity by this approach.

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