Journal of Chromatography, 427 (1988) 287–294
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4140

ISOLATION AND IDENTIFICATION OF THE GLUCURONIDE CONJUGATE OF 2-HYDROXYDESIPRAMINE BY PREPARATIVE LIQUID CHROMATOGRAPHY

RAYMOND F. SUCKOW*,*

New York State Psychiatric Institute, Analytical Psychopharmacology Division, 722 West 168th Street, New York, NY 10032 (U.S.A.)

and

THOMAS B. COOPER

Nathan S. Kline Institute, Analytical Psychopharmacology Division, Orangeburg, NY 10962 (U.S.A.) and New York State Psychiatric Institute, Analytical Psychopharmacology Division, 722 West 168th Street, New York, NY 10032 (U.S.A.)

(Received December 29th, 1987)

SUMMARY

A semi-preparative column liquid chromatographic procedure for the isolation and purification of milligram quantities of the glucuronide conjugate of 2-hydroxydesipramine, a major metabolite of desipramine, is presented. Urine from patients receiving desipramine was collected and passed through a column of XAD-2 resin. The methanolic extract was chromatographed on a reversed-phase octadecyl semi-preparative column followed by further purification on a silica gel column of the same dimension, yielding a product 95% pure. Fast atom bombardment and thermospray mass spectroscopy, as well as ultraviolet photodiode-array spectroscopy and hydrolysis with β-glucuronidase confirmed the identification and purity of 2-hydroxydesipramine glucuronide. This important glucuronide metabolite will be a useful tool as an authentic standard for pharmacokinetic and metabolism studies and for determining its pharmacological characteristics in laboratory animals.

INTRODUCTION

Conjugation of xenobiotics by glucuronidation constitutes a most important pathway of detoxification and elimination in humans as well as in many animal species. Glucuronides can form from compounds possessing such functional groups

*Address for correspondence: Nathan S. Kline Institute, Analytical Psychopharmacology Division, Orangeburg, NY 10962, U.S.A.

0378-4347/88/$03.50

as alcohols, phenols, carboxylic acids, amines and thiols. These conjugates are very water-soluble and are excreted in the urine, and to some extent, in the bile. Because of their hydrophilic and acidic nature, most glucuronide conjugates are thought not to penetrate the blood–brain barrier and, thus, have virtually no central nervous system pharmacologic activity [1]. A notable exception to this rule is an important metabolite of morphine, morphine-6-glucuronide [2].

Desipramine (DMI) is a widely used secondary amine tricyclic antidepressant. Its metabolic profile has been extensively studied as has its tertiary amine precursor imipramine. DMI can be metabolized by demethylation, hydroxylation and dealkylation, the predominant route being essentially species-dependent and genetically determined. In man, the preferred metabolic route appears to be hydroxylation at the 2-position forming 2-hydroxydesipramine (2-OHDMI) [3]. This metabolite is then conjugated with glucuronic acid to form 2-OHDMI glucuronide (2-OHDMI-G) and excreted. This conjugate can be found in plasma and urine of patients receiving therapeutic doses of DMI. The plasma concentration of 2-OHDMI-G usually exceeds the unconjugated form (2-OHDMI) by a factor of 20. In patients with chronic renal failure, this ratio is reported to be five to fifteen times higher than normal [4]. Furthermore, there is evidence that 2-OHDMI-G has peripheral pharmacological activity, but limited quantities of this material has precluded adequate testing [5].

Methods for analysis and isolation of glucuronic acid conjugates have recently been reviewed [6]. Dixon et al. [7] have successfully isolated relatively pure glucuronides of oxazepam, levorphanol and hydroxyethylflurazepam by preparative high-performance liquid chromatography (HPLC). Therefore, we decided to investigate the feasibility of isolating milligram amounts of pure 2-OHDMI-G from urine of patients receiving DMI which could subsequently be used as both an analytical and pharmacological standard. By utilizing semi-preparative HPLC, significant quantities of pure metabolite could be isolated without a time-consuming and often difficult chemical synthesis.

EXPERIMENTAL

Apparatus

All chromatography was carried out using two Model 510 solvent delivery pumps, a Model U6K sample injector (using a 2-ml sample loop), a Model 660 gradient programmer and a Model 441 UV absorbance detector at a fixed wavelength of 254 nm (all from Waters Assoc., Milford, MA, U.S.A.). Peak analysis was further assessed using a Model 1040M photodiode-array detector interfaced with the Series 300 analytical work station and a Model 7440 ColorPro plotter (Hewlett-Packard, Palo Alto, CA, U.S.A.). Chromatograms were recorded on an Omniscribe strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). Analytical separations were achieved using a Partisil-10, a Partisil-10 ODS-3 (Whatman Chemical Separation, Clifton, NJ, U.S.A.) and an LC-1 5 μm particle size reversed-phase column (Supelco, Bellefonte, PA, U.S.A.), all 25 cm $\times$ 4.6

mm I.D. Semi-preparative separations were carried out with a Partisil-10 and a Partisil-10 ODS-3 Magnum-9 column, each 50 cm in length (Whatman Chemical Separations).

Mass spectral analysis was performed on a Kratos MS-50 mass spectrometer equipped for fast atom bombardment (FAB) operating at an accelerating voltage of 8 kV. The sample was dissolved in methanol in a matrix of thioglycerol and bombarded with xenon at 8 kV. These determinations were carried out at the Middle Atlantic Mass Spectroscopy Laboratory, a National Science Foundation shared instrument facility (Baltimore, MD, U.S.A.). Liquid chromatography–mass spectroscopy (LC–MS) was done on a Hewlett-Packard Model 5988 thermospray system equipped with an electron filament operating at 900 V. A 10-μl sample was injected into a Model 1090L liquid chromatograph (Hewlett-Packard) and carried through on a mobile phase of methanol – 0.1 *M* ammonium acetate (1:1).

Reagents

All chemicals were of analytical-reagent grade. Methanol, acetonitrile and methyl *tert.*-butylether, obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.), were all HPLC-grade. β-Glucuronidase (Glusulase) was obtained through NEN Research Products (Dupont, Boston, MA, U.S.A.).

Sample preparation

Urine was collected from patients receiving DMI daily and stored refrigerated. A 200-ml aliquot of urine was first alkalinized by the addition of 1 ml of ammonium hydroxide and extracted with an equal volume of methyl *tert.*-butyl ether in a separatory funnel to remove the parent compound and other basic metabolites as well as other extractable endogenous material. The extract was discarded and the urine passed through a Whatman No. 1 filter. The urine was then neutralized with 6 *M* hydrochloric acid and passed through a column (90 cm × 3 cm) partially filled (30 cm) with Amberlite XAD-2 resin. The column was washed once with 100 ml of water followed by 200 ml of methanol. The first 100 ml of methanol extract were collected and dried down to an oily residue. A 5-ml volume of water–acetonitrile (90:10) was added to this residue and a 1–2 ml aliquot was injected into the liquid chromatograph for preparative chromatography.

Chromatography

Preparative HPLC was carried out following initial work-up on an analytical scale using the same reversed-phase or normal-phase column packing material. Once these conditions were optimized, scaling-up to a semi-preparative procedure was performed using a dual-solvent gradient system. For reversed-phase semi-preparative HPLC, 1–2 ml of extract was injected on Partisil-10 ODS-3 Magnum-9 column and eluted using gradient curve No. 7 on the Model 660 programmer. Solvent A was water–acetonitrile (90:10) and solvent B was water–acetonitrile (70:30). The gradient was run from 0 to 95% B over 30 min at a flow-rate of 5 ml/min. Initially 1-min fractions were collected to ascertain the retention time of 2-OHDMI-G. Each fraction was hydrolyzed with β-glucuronidase (Gluculase) for 18 h at room temperature and analyzed for the aglycone 2-

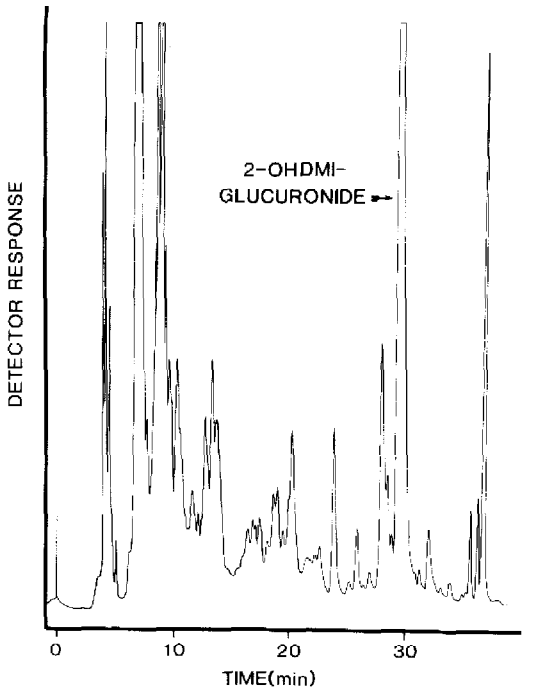
OHDMI by modifying a previously published method [8]. Once the retention time was established for the glucuronide, only that fraction was subsequently collected. This fraction was then evaporated to dryness in a vacuum centrifuge (Model SVC-100H, Savant instruments, Farmingdale, NY, U.S.A.). Additional purification was performed using a Partisil-10 silica column and a mobile phase of methylene chloride–methanol–acetic acid (70:30:1) at a flow-rate of 5 ml/min. As before, all fractions were collected and checked for the presence of 2-OHDMI-G by hydrolysis. This fraction was then evaporated to dryness as before.

A white amorphous powder was obtained and submitted for FAB-MS, thermospray LC–MS and UV photodiode-array spectroscopy as described previously.

Purity of the isolated compounds was checked by hydrolytic quantitative conversion to the aglycone with β-glucuronidase. The converted aglycone, 2-OHDMI, was quantitatively compared to a reference standard of 2-0HDMI obtained through the National Institute of Mental Health, Neurosciences Research Branch.

RESULTS

This report describes the feasibility of preparative liquid chromatography in the isolation and purification of milligram quantities of the glucuronide conjugate



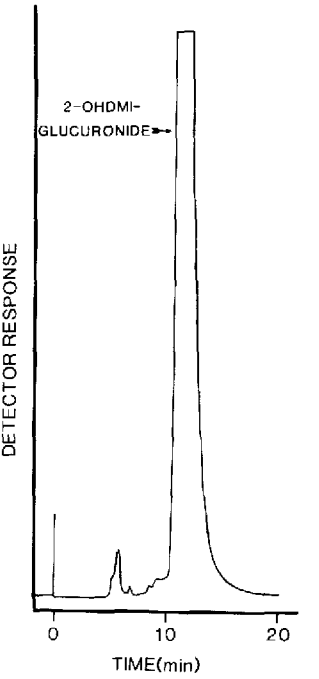


Fig. 1. Initial preparative chromatogram of a 2-ml crude urine extract injected on Partisil-10 ODS-3, 50 cm × 9.4 mm I.D. Chromatographic conditions are described in text.

Fig. 2. Final preparative chromatogram of 2-OHDMI-G injected on Partisil-10 silica, 50 cm × 9.4 mm I.D. Chromatographic conditions are described in text.

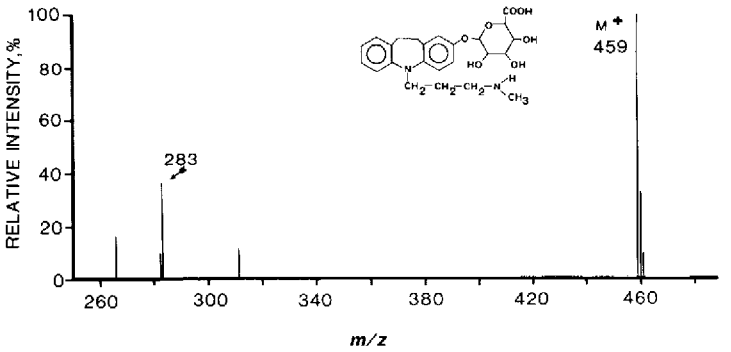


Fig. 3. Positive-ion fast atom bombardment mass spectrum of 2-OHDMI-G.

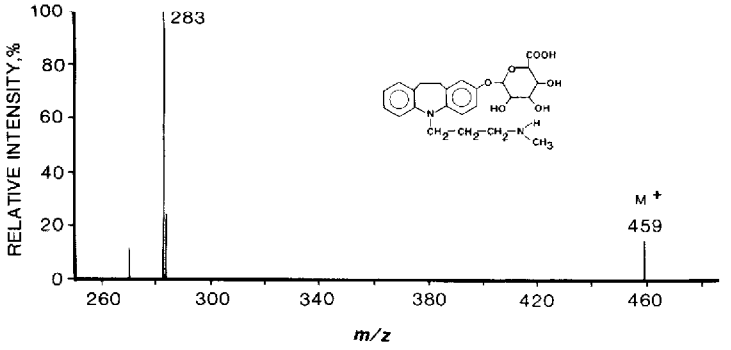
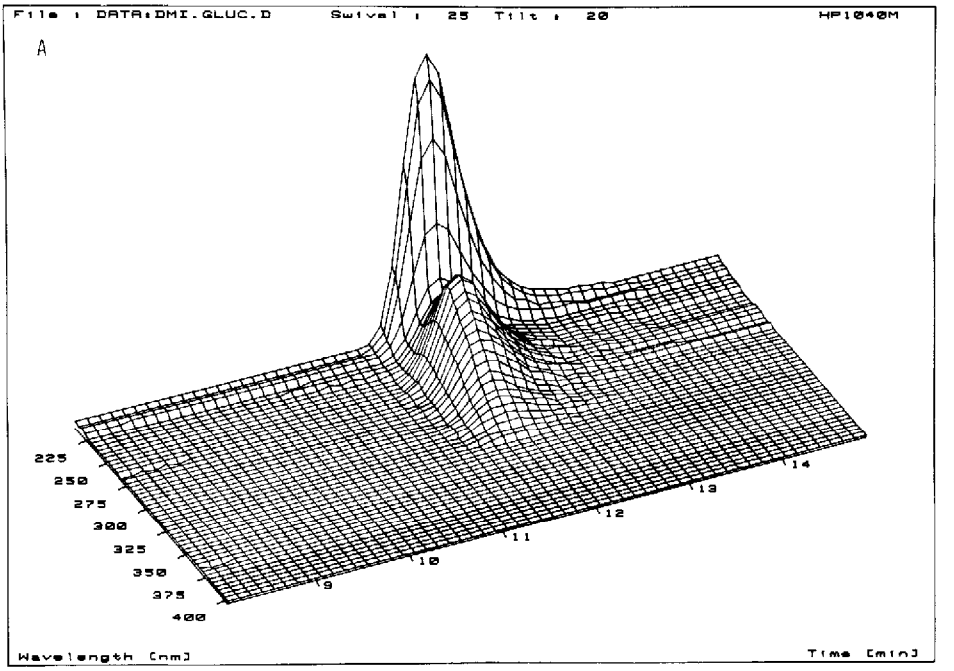


Fig. 4. Thermospray mass spectrum of 2-OHDMI-G.

of 2-OHDMI from patient urine. Fig. 1 illustrates a chromatogram of the crude urine extract injected onto a reversed-phase octadecyl semi-preparative column. Using a simple preprogrammed gradient with various concentrations of acetonitrile in water over 30 min, the glucuronide was identified to elute at about 30 min with most of the chromogenic material eluting earlier. Re-chromatography was found to be necessary in order to remove traces of pigmented material and other contaminants. This was achieved using a silica semi-preparative column as described previously. Fig. 2 shows the chromatogram of the glucuronide conjugate using the silica column. This peak was collected and concentrated, and, upon evaporation, yielded a white amorphous powder.

MS analysis performed on this compound was consistent with the structure of the glucuronide conjugate of 2-OHDMI. FAB-MS showed a positive molecular ion at *m/z* 459 (Fig. 3). Some fragmentation occurred at *m/z* 283 (MH−176) indicating the formation of the aglycone 2-OHDMI. Thermospray LC–MS on the same sample produced a molecular ion at *m/z* 459. The base peak at *m/z* 283 (MH−176) is again indicative of the formation of 2-OHDMI, the aglycone (Fig. 4). No further fragmentation occurred, nor were any other peaks observed in this spectra.

The purity of the isolated 2-OHDMI-G was further assessed by a quantitative hydrolysis procedure. Conversion of 500 ng of the isolated 2-OHDMI-G yielded



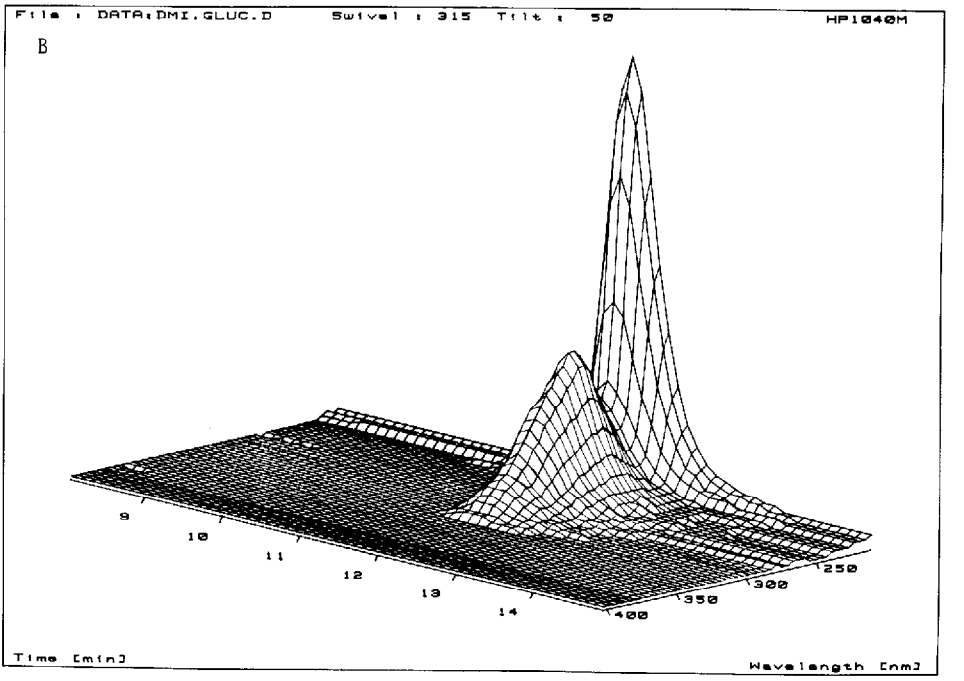


Fig. 5. (A) Photodiode-array plot of isolated 2-OHDMI-G. Chromatography performed on Partisil-10 ODS-3 (25 cm × 4.6 mm I.D.) using water–acetonitrile (80:20) at a flow-rate of 1.5 ml/min. (B) The same chromatogram viewed from a different angle.

290 ng of the aglycone, equivalent to 95% pure, assuming 100% hydrolysis with β-glucuronidase. It is interesting to note that there were no significant differences in the efficiency of the hydrolysis with β-glucuronidase between 4 and 24 h at room temperature or at 37°C.

Further analysis of the isolated compound using UV photodiode-array detection indicates the glucuronide conjugate to be pure when compared to the UV scan of pure 2-OHDMI (the glucuronic acid moiety does not appreciably absorb over 200 nm). A three-dimensional plot of a segment of the chromatogram is shown in Fig. 5A and B.

The stability of the pure 2-OHDMI-G was checked periodically. There was no appreciable deterioration of 2-OHDMI-G when stored at 4°C for up to eight months. However, aqueous solutions of this conjugate yielded up to 10% of the aglycone after six months at 4°C.

DISCUSSION

Recent technological advances in HPLC have made the purification of preparative amounts of polar conjugates generally more convenient than previous techniques. Reversed-phase HPLC has especially been popular for purification of polar conjugates [9]. Further purification by an additional normal-phase chromatographic procedure has also been reported [7]. In this study, it was necessary to employ both types of chromatography to completely separate the glucuronide from other contaminants, namely the chromogenic material found in abundance in urine. This was shown to be true when an initial MS analysis was done on a sample not re-chromatographed on silica. Several intense, but uncharacteristic peaks appeared in the mass spectrum that could not be rationalized from the chemical structure. Further purification on the silica column was, therefore, found to be necessary to achieve a purity of 95%.

FAB-MS and thermospray LC–MS are relatively recent methods in MS analysis. Both these modes are extremely useful in characterizing polar compounds such as the glucuronide metabolites. Conventional electron-impact and, to some extent, chemical ionization MS typically fragment these polar and thermally labile compounds such that the molecular ion is never seen. Trimethylsilyl derivatization of the glucuronides has been one approach to enhance volatility and increase thermal stability of these compounds [10–12]. Since both FAB-MS and thermospray LC–MS permit molecular mass determinations and fragmentation patterns to be acquired from underivatized conjugated compounds, these methods were incorporated in this study. The primary reason for using both techniques was for confirmation purposes only, and not as a comparison between these two modes. Fenselau et al. [13], however, have reported, in a recent study, that the thermospray technique produced comparatively more fragmentation than FAB on a few selected compounds. Moreover, it should be emphasized that fragmentation is a result of instrument-controlled conditions and the fragmentation pattern can change dramatically for a compound by changing instrument settings [14].

A recent report [15] remarked that the absolute yield of the hydrolysis of 2-OHDMI-G in urine could not be determined because of the unavailability of the

pure glucuronide as a standard. This together with the report that 2-OHDMI-G has peripheral pharmacological activity which could not be further substantiated because of limited availabilities [5] emphasizes the importance of a method for isolating and purifying this compound. This procedure can produce 2-OHDMI-G in sufficient quantity necessary for analytical standards as well as pharmacological testing both in vitro and in vivo in animals to determine what role, if any, this compound may have in peripheral side-effects associated with treatment with DMI. Clinical pharmacokinetics of DMI may now be further investigated in terms of the formation and disposition of 2-OHDMI-G without the need for uncontrolled hydrolysis to the aglycone.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Catherine Fenselau of the Middle Atlantic Mass Spectroscopy Laboratory and to Dr. Jim Lau of Hewlett-Packard Co. for their valuable assistance in performing the FAB-MS and thermospray MS, respectively. This project was supported in part by BRSG-05650 awarded by the Biomedical Research Grants Program, NIH, and by MHCRC 30906.

REFERENCES

1 R.L. Smith and R.T. Williams, in G.J. Dutton (Editor), Glucuronide Acid Free and Combined, Academic Press, New York, 1966. 457–491.
2 H. Yoshimura, S. Ida, K. Oguri and H. Tsukamoto, Biochem. Pharmacol., 22 (1973) 1423.
3 J.L. Crammer, B. Scott and B. Rolfe, Psychopharmacologia (Berlin), 15 (1969) 207.
4 J.A. Lieberman, T.B. Cooper, R.F. Suckow, H. Steinberg, M. Borenstein, R. Brenner and J.M. Kane, Clin. Pharmacol. Ther., 37 (1985) 301.
5 V.W. Theobald, O. Buch, H.A. Kunz and C. Murpurgo, Med. Pharmacol. Exp., 15 (1966) 187.
6 J. Tomasic, in E.R. Garrett and J.L. Hirtz (Editors), Drug Fate and Metabolism, Vol. 2, Marcel Dekker, New York, 1978, pp. 281–335.
7 R. Dixon, R. Evans and T. Crews, J. Liq. Chromatogr., 7 (1984) 177.
8 R.F. Suckow and T.B. Cooper, J. Pharm. Sci., 73 (1984) 1745.
9 W. Muecke, in G.D. Paulson, J. Caldwell, D.H. Hutson and J.J. Menn (Editors), Xenobiotic Conjugation Chemistry, American Chemical Society, Washington, DC, 1986, pp. 108–123.
10 T.T.L. Chang, C.F. Kuhlman, R.T. Schillings, S.F. Sisenwine, C.O. Tio and H.W. Ruelius, Experientia, 29 (1973) 653.
11 S. Billetts, P.S. Lietman and C. Fenselau, J. Med. Chem., 16 (1973) 30.
12 C. Fenselau and L.P. Johnson, Drug Metab. Dispos., 8 (1980) 274.
13 C. Fenselau, D.J. Liberato, J.A. Yergay, R.J. Cotter and A.L. Yergay, Anal. Chem., 56 (1984) 2759.
14 W.H. McFadden and S.A. Lammert, J. Chromatogr., 385 (1987) 201.
15 E. Spina, A. Arena and F. Pisani, Ther. Drug Monit., 9 (1987) 129.