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MEASUREMENT OF THE FORMATION OF MENTHOL GLUCURONIDE *IN VITRO*, BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER PRE-COLUMN LABELLING WITH 4-BROMOMETHYL-7-METHOXYCOUMARIN

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SUMMARY

Menthol glucuronide was measured in the presence of borneol glucuronide as internal standard by a high-performance liquid chromatography method including a pre-column derivatization step. The carboxylic group of glucuronic acid conjugates was esterified by 4-bromomethyl-7-methoxycoumarin with potassium carbonate and 18-crown-6-ether in acetone. The resulting esters were chromatographed on a reversed-phase column (LiChrospher CH-18) with methanol–water (75:25, v/v) as eluant. They were detected by UV spectrophotometry (λ = 328 nm), the sensitivity being 10^{-11} mol of each derivatized glucuronide injected. Several parameters have been studied in order to optimize the derivatization procedure. The method was applied to the determination of glucuronyl transferase activity towards menthol during *in vitro* assays.

INTRODUCTION

Glucuronidation is the most common conjugation pathway occurring during the metabolism of endogenous and foreign substrates. Glucuronides are produced in the presence of uridine diphosphoglucuronic acid (UDPGA) and UDP glucuronyl transferase (UDPGT; E.C. 2.4.1.17). This enzymatic system is mainly localized in hepatic microsomes and the resulting polar conjugates are excreted in urine. The factors influencing UDPGT activity are usually investigated by *in vitro* assays[1,2].

The analysis of glucuronides in biological fluids has been extensively described. Traditionally, the aglycon is released by either acidic or enzymatic hydrolysis and is measured before and after this step; the difference between the results affords the conjugation rate. However, these indirect methods lack specificity and errors may occur because of incomplete hydrolysis or loss of aglycon. For these reasons, increasing use is being made of direct analysis of glucuronides. The developments in gas chromatography–mass spectrometry (GC–MS)[3], immunologic assays[4] and high-performance liquid chromatography (HPLC)[5] for the determination of glucuronides have been reviewed.

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In GC systems, glucuronides have to be converted into volatile derivatives, but GC together with MS affords the best possibility for structural studies. Direct quantitation of glucuronides has been achieved by various immunologic and HPLC methods. The main difficulty encountered in the development of immunologic assays consists in the production of specific antibodies directed towards the measured glucuronide, in order to avoid cross-reactions with the aglycon and/or other metabolites[6]. HPLC techniques do not suffer from this disadvantage since glucuronides can easily be separated from their respective aglycon and other conjugates, as shown in a study of acetaminophen metabolism[7].

The detection of glucuronides in HPLC systems generally relies upon the spectral or electrochemical properties of the aglycon, since the sugar moiety of these compounds exhibits only a weak UV absorbance at about 200 nm due to the carbonyl group. So, an HPLC assay cannot be used for glucuronides whose aglycon has no chromophore, such as aliphatic alcohols and terpenoid substances.

Sensitive methods using radiolabelled drugs conjugated with glucuronic acid have been reported[8,9] but are costly and time-consuming. Derivatization is another way of lowering the detection limit. In a procedure applied to steroid conjugates[10], the reaction site is a chemical group of the aglycon and this latter fact does not permit an extension of the procedure to any glucuronide, as would be the case of a reaction involving the carboxylic group of glucuronic acid.

Many techniques have been described for the derivatization of carboxylic acids[11]. Among them, esterification with 4-bromomethyl-7-methoxycoumarin (BrMmc)[12] has been applied to fatty acids[13], protaglandin[14] and drugs[15]. The resulting esters are easily eluted on reversed-phase columns with water–alcohol mixtures without addition of salts or counter ions. We have now applied this method to the determination of menthol glucuronide. Several parameters were tested in order to optimize the reaction. The procedure was then applied to the measurement of the conjugate produced *in vitro* in the presence of microsomal proteins.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents used were of analytical reagent grade. Menthol, 4-bromomethyl-7-methoxycoumarin (BrMmc) and 18-crown-6-ether were purchased form Fluka (Buchs, Switzerland). Menthol glucuronide ammonium salt, Tris–HCl and Triton X-100 were obtained from Sigma (St. Louis, MO, U.S.A.). Uridine diphosphoglucuronic acid (UDPGA) sodium salt was supplied by Boehringer (Mannheim, F.R.G.) and benzonaphthol by Cooper (Melun, France).

Borneol glucuronide was synthesized by a Helferich reaction using stannic chloride as the catalyst and the same conditions as those previously reported for other aglycons[16]. The IR and NMR spectra for the glucuronide obtained were recorded and its purity (over 98%) was determined by potentiometric titration[17].

Preparation and activation of microsomal fractions

Hepatic microsomal fractions were obtained from male Sprague–Dawley rats pretreated with phenobarbital, by a conventional ultracentrifugation technique, and their protein content was measured by a colorimetric method[2]. The microsomes were

frozen (−20°C) and used within 4 weeks. They were diluted in a 75 mM Tris–HCl buffer pH 7.4 containing 5 mM magnesium chloride, to a final protein concentration of 2.5 mg/ml; Triton X-100 was added to the microsomal suspension in a detergent: protein ratio value of 0.4 (w/w). The mixture was allowed to stand for 20 min at 0°C, to complete activation.

Determination of enzymatic activity

The assays were performed in 15-ml flasks fitted with a screw cap and protected from light by aluminium foil. A 0.15-ml volume of the activated microsomal preparation (see above) was mixed with 0.03 ml of a 100 mM aqueous solution of UDPGA. The mixture was pre-incubated in a shaking water-bath at 37°C for 5 min before addition of various volumes (0.005–0.08 ml) of a 1 mg/ml menthol solution prepared in 40% (v/v) ethanol. The final reaction volume was adjusted to 1.0 ml with the Tris–HCl buffer pH 7.4 containing magnesium chloride.

The mixture was then incubated in a shaking water-bath at 37°C for 20 min. The reaction was stopped by adding 0.15 ml of 0.15 M hydrochloric acid and by transferring the flask to an ice-bath.

A 0.2-ml volume of 0.5 mg/ml borneol glucuronide solution in 1.5 mM aqueous sodium hydroxide and 1.5 ml of 0.1 M phosphate buffer pH 2.5 were added to the mixture. The conjugates were extracted by using diethyl ether (2 × 5 ml) and vigorous shaking. The organic layers were collected, dehydrated over sodium sulphate and evaporated to dryness under a nitrogen stream at a temperature less than 40°C.

Derivatization procedure

The procedure was optimized by varying the reaction time, temperature and BrMmc excess over the ranges indicated in Fig. 2. Each reaction mixture contained 0.1 mg of menthol glucuronide, 0.5 mg of 18-crown-6 ether and 20 mg of anhydrous potassium carbonate. A 0.02-ml volume of a 0.1 mg/ml benzonaphthol (internal standard) solution in acetone was added to each assay.

The conditions selected for biochemical studies were as follows: the dried residue obtained after the extraction step was redissolved in 1 ml of acetone and 0.5 ml of 1.5 mg/ml BrMmc solution, 0.5 ml of 1 mg/ml 18-crown-6 ether solution and 20 mg of potassium carbonate were added. The reaction mixture was heated at 70°C for 30 min, then cooled in ice and an aliquot injected into the chromatograph.

Chromatographic conditions

The HPLC system consisted of a ternary solvent-delivery pump (Model SP 8700; Spectra-Physics, Santa Clara, CA, U.S.A.), an injection value with a 10-μl sample loop (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and a UV–visible detector (Model LC 871; Pye Unicam, Cambridge, U.K.). The reversed-phase columns were prepacked with LiChrospher CH-18 (Hibar R.T. 250-4, 5 μm; E. Merck, Darmstadt, F.R.G.). A pre-column (25 × 4 mm I.D.) packed with LiChrosorb RP-18 (7 μm) was used on-line for all chromatographic analyses. The mobile phases were methanol–water (80:20, v/v) for the optimization procedure and 75:25 (v/v) for the biochemical assays. They were filtered through a 0.6-μm microfilter (Type HVLP; Millipore, Bedford, MA, U.S.A.) and deaerated before use. The flow-rate was 1.5

ml/min. The spectrophotometric detector was set at 240 nm for the optimization of the derivatization procedure and at 328 nm for the biochemical studies. The chromatograms were recorded and all calculations made with an integrator (Model 5020, Spectra-Physics).

RESULTS AND DISCUSSION

Analytical development of the method

The reaction scheme in Fig. 1 shows the esterification in the presence of potassium carbonate, acting as a base, and 18-crown-6-ether. The latter complexes the K^+, whereas the anionic form of the glucuronide is attacked by the halogenated reagent (BrMmc), producing the methyl methoxycoumarin (Mmc) glucuronide ester.

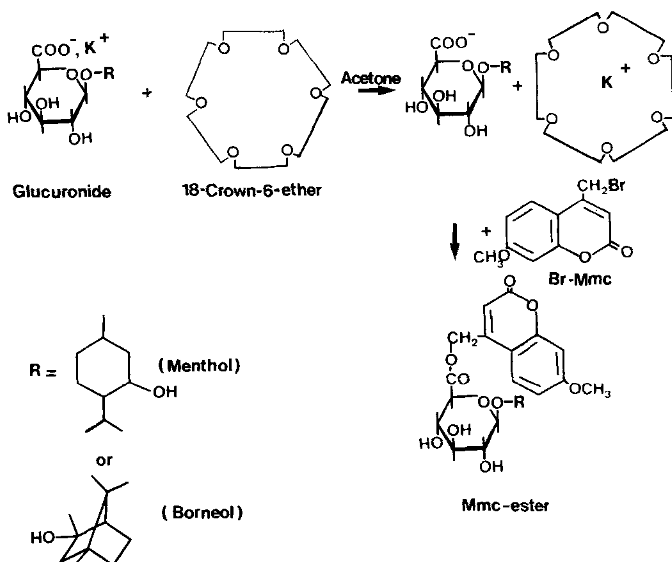

Fig. 1. The esterification of the menthol or borneol glucuronide with 4-bromomethyl-7-methoxycoumarin (BrMmc) in the presence of potassium carbonate and 18-crown-6-ether, in acetone as solvent.

The optimum conditions for this esterification have been determined by varying the temperature, reaction time and reagent excess (Fig. 2). For this purpose, benzonaphthol was used as the internal standard. Its capacity factor, k', was 7.3 under the chromatographic conditions described in the Experimental section. The conditions selected (reaction time 30 min, heating temperature 70°C) are close to those previously reported for the derivatization of fatty acids with BrMmc[13]. A two-fold excess of the reagent over the initial amount of glucuronide is enough to ensure the maximum yield of ester, but the presence of other organic acids, such as UDPGA, in biological fluids has to be considered. The ratio used during biochemical studies was always greater than 5:1.

The residual water contained in the reaction mixture has a considerable effect on the esterification rate. Even a small proportion of water added to anhydrous

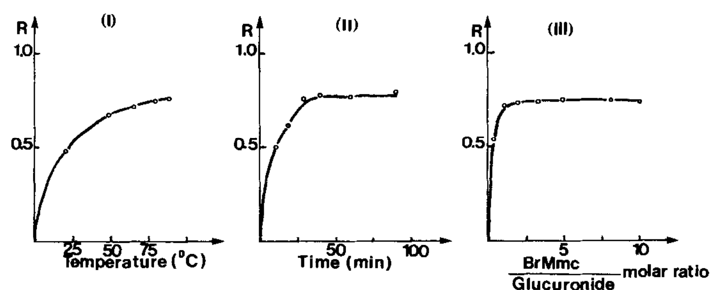

Fig. 2. Rate of esterification of the menthol glucuronide with BrMmc as a function of the temperature (I), time (II) and reagent excess (III). *R* is the ratio of the peak areas for Mmc-menthol glucuronide ester and benzonaphthol used as internal standard.

acetone, *i.e.*, 3% (v/v), results in a decrease in yield of the ester. So the derivatization step has to be carried out on a dried extract of the biological sample. The extraction of the incubation mixture with an organic solvent permits the isolation of both menthol and borneol glucuronides, the latter being used as internal standard. Their recovery was calculated to be about 80%.

The stability of the Mmc glucuronide esters was tested by keeping the reaction mixture in ice for 2 h. By this time, no significant variations appeared upon periodical injections of the solution into the chromatograph.

The use of UV spectrophotometry for the detection of Mmc glucuronide esters affords a sensitivity in the range of 10 pmol of each derivatized glucuronide injected into the chromatographic system. Fluorimetric detection of these adducts would afford a lower limit.

In vitro studies of the UDP-glucuronyl transferase activity

The method described was applied to the measurement of menthol glucuronide produced during *in vitro* incubation in the presence of microsomal proteins in order to determine the UDPGT activity. As seen in Fig. 3, the peak corresponding to the menthol glucuronide derivative is well separated from other peaks due to the internal standard, derivatizing agents and constituents of the incubation mixture. The menthol glucuronide was quantitated by use of a calibration curve constructed from the results for incubation solutions free from menthol and spiked with known amounts of the standard menthol glucuronide. Good linearity was obtained in the range from $1 \cdot 10^{-5}$ to $14 \cdot 10^{-5}$ *M*. The accuracy of the method, including the extraction, derivatization and HPLC steps, was calculated over five replicate assays; the coefficient of variation was 4.5%.

The UDPGT activity of microsomal fractions was measured as a function of the protein concentration and incubation time. Glucuronide production proceeded linearly in the range of 0.1 to 0.8 mg of protein per ml and up to an incubation time of 30 min.

The effect of the menthol concentration on the formation rate of its glucuronide was tested under constant conditions, 0.375 mg of protein per ml and 20 min of incubation. This UDPGT activity measurement showed substrate inhibition when the menthol concentration was greater than 30 m*M*. The Lineweaver–Burk plot (Fig.

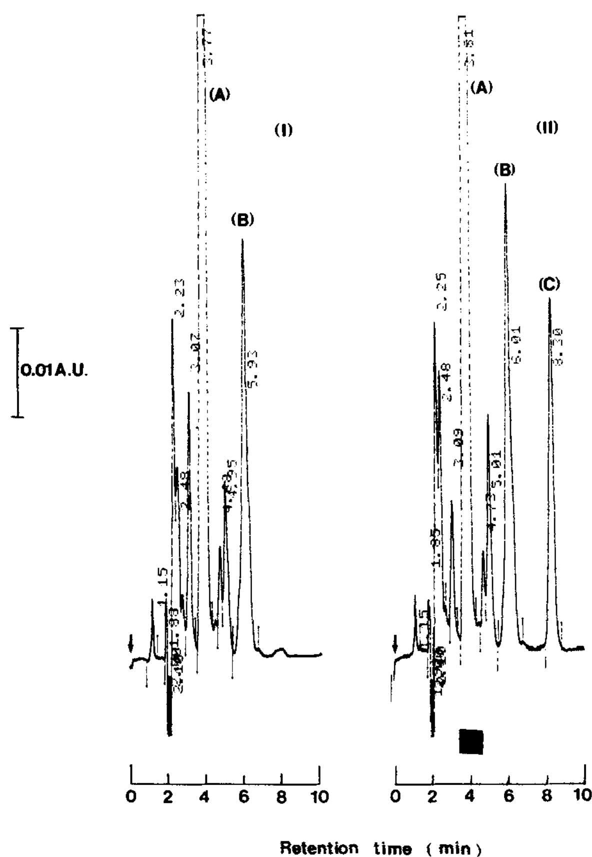

Fig. 3. Chromatograms of glucuronides extracted from microsomal reaction mixtures, derivatized with BrMmc and eluted on a LiChrospher CH-18 (5 μm) column (250 × 4 mm) with methanol–water (75:25, v/v) at a flow-rate of 1.5 ml/min. Detection at 328 nm. Peaks: A = BrMmc; B = Mmc-borneol glucuronide ester; C = Mmc-menthol glucuronide ester. (I) Blank resulting from a microsomal incubation containing no menthol, with borneol glucuronide as internal standard. (II) As (I) but with $2.8 \cdot 10^{-7}$ mol of menthol added before incubation.

4) still permitted the calculation of the Michaelis constant, K_M = 0.29 mM, and the maximum velocity, $V_{max.}$ = 16.2 nmol/min per mg of protein. These kinetic data were deduced from the intercepts with the x and y axes of the straight line corresponding to the production of glucuronide *versus* substrate concentration. These biochemical parameters are in accord with values previously reported for the same substrate and measured by a modified Mulder and Van Doorn kinetic procedure[2].

The described direct method for measurement of menthol conjugated with glucuronic acid is of interest in the study of UDPGT polymorphism, and terpenoid substrates permit some differentiation in this enzymatic system[2].

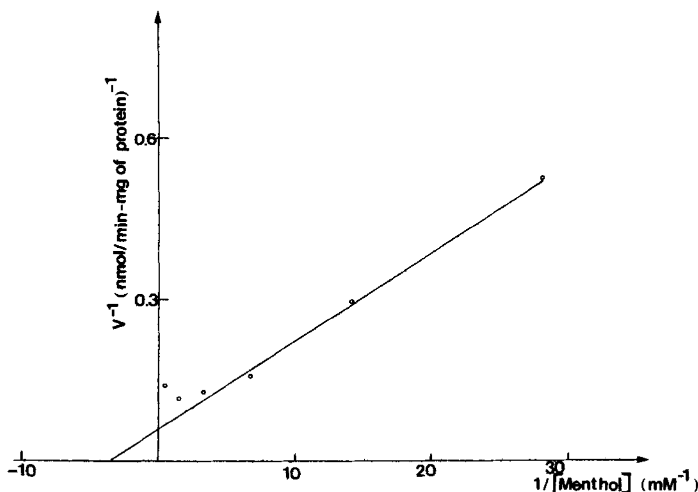

Fig. 4. Lineweaver–Burk plot of glucuronyl transferase activity towards menthol. The kinetic data were obtained by measuring the concentration of menthol glucuronide produced as a function of substrate concentration under constant conditions: 0.375 mg/ml of microsomal proteins and 20 min of incubation.

CONCLUSIONS

Other HPLC methods have been described for the measurement of glucuronides produced in microsomal reactions[18–20], using direct detection with spectrophotometric[18,19] or amperometric methods[20] based on the aglycon properties. So far there has been no report of menthol glucuronide analysis by HPLC. The use of a chromogenic reagent for labelling this conjugate provides a selective and sensitive way of detection in a chromatographic system. The method described is now being applied to other glucuronides in our laboratory.

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