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ISOLATION, PARTIAL IDENTIFICATION AND QUANTITATIVE DETERMINATION OF FOUR GUAIPHENESIN GLUCURONIDES IN PLASMA AND URINE OF THE HORSE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The isolation, partial identification and quantitative determination of four guaiphenesin glucuronides in plasma and urine of the horse is described. The identity of the glucuronides was checked by UV and fluorescence spectrophotometry, by NMR spectrometry and by mass spectrometry after permethylation. The applicability of the procedure to pharmacokinetic studies is demonstrated.

INTRODUCTION

Guaiphenesin [3-(2-methoxyphenoxy)-1,2-propanediol] is a centrally acting muscle relaxant and we are interested in its fate in the horse. Earlier we described a method for the quantitative determination of this drug and its main metabolite, β-(2-methoxyphenoxy)lactic acid, in plasma by high-performance liquid chromatography (HPLC)[1]. For measuring the concentration of the drug and its metabolites in horse urine we had to develop another method, able to differentiate between the various metabolites. By this method we found that guaiphenesin does not appear in urine unchanged, but either in the conjugated form, mainly as glucuronides, or as the metabolite β-(2-methoxyphenoxy)lactic acid, which appears only as the unconjugated free form in urine.

To study the chromatographic behaviour of the glucuronides and for calibration we had to isolate these compounds from horse urine after a high dose of guaiphenesin.

EXPERIMENTAL

Materials

Guaiphenesin was obtained from OPG (Utrecht, The Netherlands). The metabolite β-(2-methoxyphenoxy)lactic acid was isolated from horse urine after an

* Deceased.

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i.v. injection of guaiphenesein in a dose of 100 mg/kg[1]. O-Desmethylnaproxen was kindly supplied by Syntex Laboratories (Palo Alto, CA, U.S.A.). LiChrosorb RP-18 (5 μm) and *p*-nitrophenol glucuronide were obtained from E. Merck (Darmstadt, F.R.G.), *o*-hydroxyhippuric acid from Merck-Schuchardt (Hohenbrunn, F.R.G.) and naphthoresorcinol (1,3-dihydroxynaphthalene) from Janssen Chimica (Beerse, Belgium). The β-glucuronidase used was type VII, obtained from Sigma (St. Louis, MO, U.S.A.). The anion-exchange resin Servachrom XAD-2 (100–200 μm) was obtained from Serva (Heidelberg, F.R.G.) and the methylating agents iodomethane and trideuteroiodomethane from Riedel de Haën (Seelze-Hannover, F.R.G.) and Janssen Chimica, respectively. All other chemicals were of analytical-reagent grade.

Apparatus

A Hewlett-Packard 1084B high-pressure liquid chromatograph with an automatic sampling system was used. The column was a 15 cm × 4.6 mm I.D. stainless-steel tube packed by the slurry technique with LiChrosorb RP-18 (5 μm); the column was operated at 35°C.

Isolation of guaiphenesin glucuronides

Urine was collected from a horse after 3 i.v. infusions of guaiphenesin (total 350 mg/kg). After extraction with diethyl ether, 25 ml of urine were applied to the XAD-2 column. The column was washed with 0.1 *N* hydrochloric acid and water to remove the salts, then the glucuronides were eluted with methanol. The methanolic solution was injected into the HPLC system. The elution solvent was methanol–citrate buffer (pH 6.5) (10:90) (final concentration 0.01 *M*). This methanolic solution was brought to dryness. Four peaks detected with the UV detector and suspected of representing glucuronides of guaiphenesin were collected in one combined fraction, which subsequently was processed on the XAD-2 column in order to remove the citrate from the eluting solvent. The resulting pure methanolic solution was brought to dryness. The residue contained the four presumed glucuronides. Their identities were verified by UV spectrophotometry, fluorescence spectrophotometry, nuclear magnetic resonance (NMR) spectroscopy, gas chromatography–mass spectrometry after permethylation both with iodomethane and with trideuteroiodomethane[2] and the colour reaction with naphthoresorcinol reagent[3]. For some of these tests *p*-nitrophenol glucuronide was used as a reference compound. The glucuronides were also treated with β-glucuronidase and hydrochloric acid to hydrolyse them to guaiphenesin. The concentrations of guaiphenesin glucuronides and guaiphenesin in the samples were measured by HPLC before and after the treatments.

Sample preparation

Plasma. In a centrifuge tube with a PTFE-lined screw-cap were placed 500 μl of plasma, 100 μl of the internal standard solution (O-desmethylnaproxen, 2.5 mg/ml in methanol) and 400 μl of acetone and 10 min after homogenization the sample was centrifuged for 15 min at 1000 *g*. The supernatant was transferred into another tube and evaporated to dryness under a stream of dry air at 35°C. The residue was taken up in 0.5 ml of elution solvent and 10 μl of this sample were injected into the HPLC system.

Urine. A 200-μl volume of 10-fold diluted urine was transferred into a sample

vial of the automatic sampling system and 600 μl of doubly distilled water and 200 μl of the internal standard solution (*o*-hydroxyhippuric acid, 3 mg/ml in water) were added. A 10-μl volume of this sample was injected into the HPLC system.

Chromatographic conditions

For plasma samples the column was connected to a Perkin-Elmer 3000 fluorescence detector. The operating conditions for maximum fluorescence were excitation wavelength 230 nm with slit 10 nm and emission wavelength 306 nm with slit 5 nm. The output of the detector was fed into a Hewlett-Packard processor. For the analysis of urine samples the built-in UV detector was used and operated at 275 nm.

The elution solvent was a mixture of methanol and citrate buffer (pH 6.5) (10:90) (final concentration 0.01 *M*). Elution was performed at a flow-rate of 2.0 ml/min.

Calibration

Known amounts of β(2-methoxyphenoxy)lactic acid were added to blank urine, and known amounts of hippuric acid were added to water. Linearity was verified by plotting the amount calculated from the peak height against the amount in urine or water.

Linearity of the four glucuronides was verified by dilution of horse urine containing the glucuronides with blank horse urine to obtain a concentration curve. The product of peak height and retention time was used as a measure of amount.

RESULTS

Isolation of glucuronides

The UV maxima (220 and 275 nm) of the glucuronide fraction were in accordance with those of guaiphenesin itself. Also, the fluorescence spectrum was the same as that of guaiphenesin. The NMR spectrum recorded in CD_3OD with trimethylsilane as the reference showed the presence of a methoxy group (3.85 ppm) and a phenoxy group (6.92 ppm) as in guaiphenesin.

The mass spectrometric fragmentation of the permethylated glucuronides was the same as described in the literature[2]. When methylation was carried out with CH_3I and with CD_3I the shift in the spectrum was exactly as expected: the M^+ peak had an *m/e* value of 444 after methylation with CH_3I and 459 after methylation with CD_3I.

The colour reaction with naphthoresorcinol reagent was positive, indicating the presence of the glucuronide moiety.

When the glucuronide fraction was hydrolysed, the increase in the guaiphenesin concentration was equal to the decrease in the glucuronide concentration, within an error of 8% (concentration measured as peak height multiplied by the retention times).

Using guaiphenesin as a standard we determined that the isolated glucuronide fraction consisted of at least 80% (w/w) of guaiphenesin glucuronides.

High-performance liquid chromatography

Typical chromatograms of a blank plasma sample and a plasma sample after

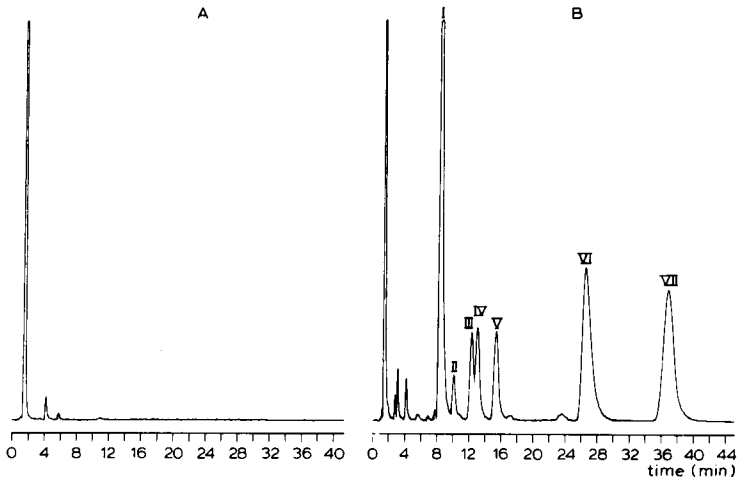

Fig. 1. High-performance liquid chromatograms from the fluorescence detector of (A) a blank plasma sample and (B) a plasma sample after a guaiphenesin dose with the peaks of β-(2-methoxyphenoxy)lactic acid (I), guaiphenesin glucuronides a–d (II–V), desmethylnaproxen (VI) and guaiphenesin (VII). After 31 min the attenuation was changed.

a guaiphenesin dose are shown in Fig. 1. The glucuronides are indicated as a, b, c and d as they elute in that order. Similar chromatograms of urine samples are shown in Fig. 2. It was verified that the large peak seen by the UV detector with a retention time of 3.9 min in blank urine was hippuric acid. Under the chromatographic conditions applied, the retention times of *o*-hydroxyhippuric acid, β-(2-methoxyphenoxy)lactic acid, guaiphenesin glucuronides a, b, c and d and O-desmethylnaproxen are about 6.2, 8.7, 10.9, 13.4, 14.2, 16.7 and 27.0 min, respectively.

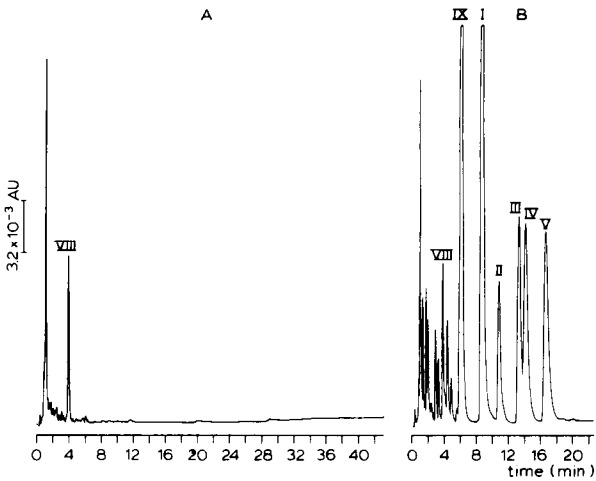

Fig. 2. High-performance liquid chromatograms from the UV detector of (A) a blank urine sample and (B) a urine sample after a guaiphenesin dose, with peaks of hippuric acid (VIII), *o*-hydroxyhippuric acid (IX), β-(2-methoxyphenoxy)lactic acid (I) and guaiphenesin glucuronides a–d (II–V).

TABLE I

CHROMATOGRAPHIC PARAMETERS OF THE COMPOUNDS

The asymmetry factor is the width of the right half of the peak divided by that of the left half, as measured at 10% of the peak height.

Compound	*No. of theoretical plates*	*Capacity factor*	*Asymmetry factor*	*Concentration range (μg/ml urine)**	*Correlation coefficient***
Hippuric acid	33,000	5.4	1.8	1000–10,000	0.9994
β-(2-Methoxy-phenoxy)lactic-acid	12,300	13.2	3.5	2500–30,000	0.9991
Glucuronide a	37,000	17.2	1.9	200–3000	0.9991
Glucuronide b	33,500	21.3	–	500–6000	0.9997
Glucuronide c	27,500	22.6	–	500–6000	0.9993
Glucuronide d	31,000	26.8	2.1	500–6000	0.9988

* The concentrations of the glucuronides are given as μg/ml of guaiphenesin in urine.
** Correlation between UV response and concentration in the sample.

Chromatographic parameters (numbers of theoretical plates, capacity factor and asymmetry factor) are given in Table I. No asymmetry factor is given for glucuronides b and c as their resolution is only 0.20. In the concentration ranges indicated in Table I, the peak heights were linear with concentration for hippuric acid and β-(2-methoxyphenoxy)lactic acid, and the peak heights multiplied the retention time were linear with concentration for the four guaiphenesin glucuronides. The correlation coefficients are also shown in Table I.

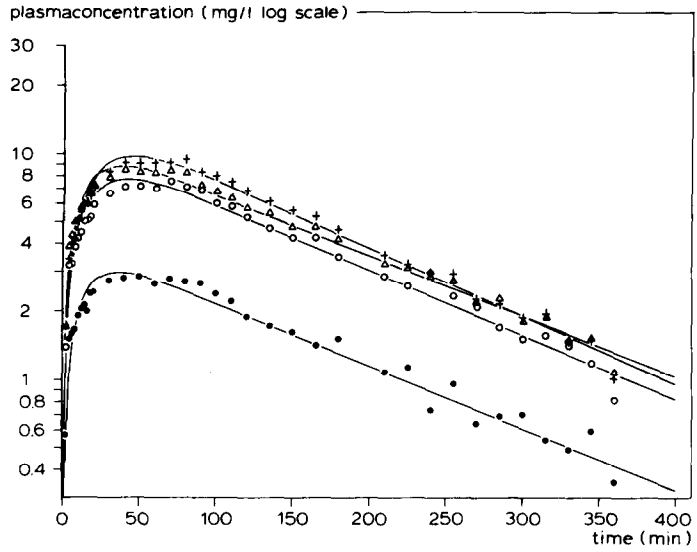

Fig. 3. Plasma profile of a horse after i,v. injection of 100 mg/kg of guaiphenesin. The concentrations of the glucuronides are given as mg/l of guaiphenesin in plasma. Key: ●, glucuronide a; ○, b; △, c; +, d.

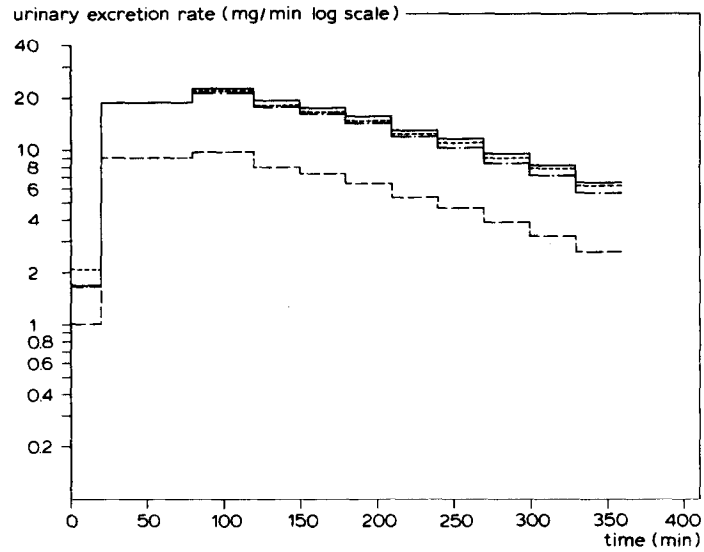

Fig. 4. Urine profile of a horse after i.v. injection of 100 mg/kg of guaiphenesin. The elimination rate is given in mg/min of guaiphenesin. Key: – – –, glucuronide a; –·–·–, b; ·····, c; ———d.

DISCUSSION

From the results of the isolation and identification of the glucuronide fraction it seems that we are dealing with glucuronide conjugates of guaiphenesin. It may be noted that the M^+ peak in the mass spectrum of the permethylated substances corresponds exactly with the theoretical molecular weight of a fully methylated guaiphenesin glucuronide (444). Obviously, our procedures could not determine the actual position of the glucuronide moiety in the molecule. Guaiphenesin contains two hydroxy groups that may be conjugated. However, four different glucuronides were found. Assuming that only monoglucuronides are formed, the most plausible explanation for this paradox is the fact that guaiphenesin has a centre of asymmetry so that each glucuronide may exist in two diastereoisomeric forms.

The chromatographic method described was applied to pharmacokinetic studies of guaiphenesin and its metabolites in horses. As a typical example, Fig. 3 shows a plasma profile of the glucuronides in a horse after an i.v. injection of guaiphenesin (100 mg/kg). From the same experiment, Fig. 4 shows the profile of urinary excretion rates of the glucuronides as a function of the time after administration of guaiphenesin. A detailed pharmacokinetic analysis of guaiphenesin and its metabolites in the horse will be reported elsewhere.

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