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Note

Rapid high-performance liquid chromatographic method for the measurement of hymecromon and its conjugates in blood plasma or serum at concentrations attained during therapy

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Hymecromon (Fig. 1, I = 7-hydroxy-4-methyl-2-H-benzopyran-2-one) is a choleretic and biliary antispasmodic drug which is metabolized in the human body mainly via glucuronidation of the 7-hydroxy function. At present only a few methods are available for the simultaneous qualitative or quantitative analysis of xenobiotics together with their glucuronide and sulphate conjugates. The wide range of polarities between the parent compounds and the highly polar conjugates makes the use of a single chromatographic separation procedure difficult.

Methods currently used to separate conjugates are reversed-phase chromatography [1], liquid—liquid counter-current partition [2], gas chromatography [3], ion-exchange chromatography [4] and ion-pair reversed-phase chromatography which has proved successful in separating steroid conjugates [5], the glucuronide and sulphate conjugates of harmol, phenolphthalein, 4-nitrophenol [6], and most recently of morphine and its 3- and 6-glucuronides [7].

Pharmacokinetics of hymecromon have received only scant attention. Assay methods currently employed include gel filtration, solvent extraction after enzymatic cleavage of the conjugates, and fluorimetry [8]. These techniques are slow and not sufficiently selective and precise.

The principle aim of this study was to investigate whether a simple isocratic reversed-phase system could be found to separate hymecromon and its major metabolites (Fig. 1). Since the compounds under investigation are ionizable in the pH range 2—8 the possibility of influencing the retention by ion-pair

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formation was studied. The developed technique is sensitive, selective, and simple to perform; an average of twenty plasma samples can be analyzed routinely during an 8-h working day.

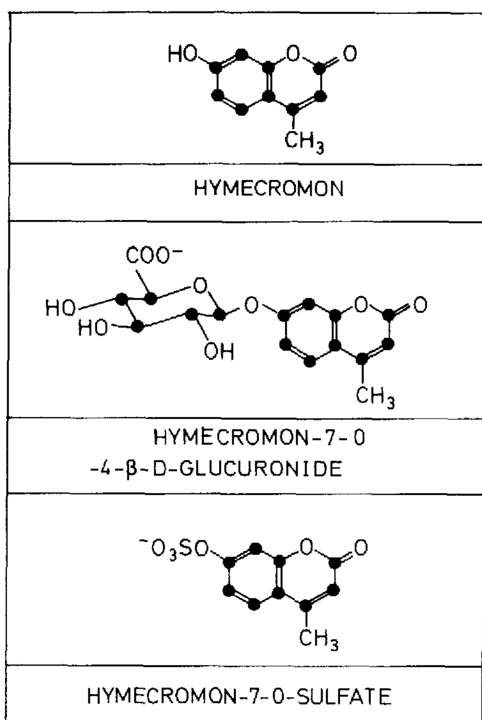

Fig. 1. Structural formulae of hymecromon and its major metabolites.

EXPERIMENTAL

Chemicals

Hymecromon was obtained from Lipha Arzneimittel (Essen, F.R.G.). Methanol (HPLC grade reagent No. 8402) was purchased from Baker (Gross-Gerau, F.R.G.). Water used for high-performance liquid chromatographic (HPLC) separations was a pyrogen-free product of Fresenius (Bad Homburg, F.R.G.). Tetrabutylammonium bromide was obtained from EGA (Steinheim, F.R.G.), 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-sulphate from Sigma (Taufkirchen, F.R.G.) and Perchlorsäure/Perchlorat No. 9431 from Merck (Darmstadt, F.R.G.).

Sample preparation

A 100-μl volume of serum (or plasma) was deproteinized by mixing with the same volume of the perchloric acid—perchlorate mixture, vigorously shaken for 30 min in an Eppendorf Mischer 5432, and centrifuged for 15 min in an Eppendorf centrifuge 5412; 10 μl of the clear supernatant was injected into the column. By addition of a constant amount of hymecromon-7-O-

sulphate it was confirmed that the recovery was the same from one patient sample to the next. So the use of an internal standard could be avoided for routine procedures.

Serum standard solutions

Serum standards containing 0.1—50 μg of compounds I—III were prepared by dissolving the compounds in appropriate volumes of drug-free serum. The further processing was the same as described under sample preparation.

Liquid chromatography

The HPLC system used was a Waters M 45 volume delivery system combined with a U6K universal injector, and a Waters 440 dual-channel ultraviolet detector with 254-nm and 280-nm filters. A stainless-steel column was packed with a stable reversed-phase stationary phase consisting of porous silica beads (mean diameter 10 μm) coated with a chemically bonded monolayer of octadecylsilane (μBondapak C_{18}, Waters Assoc.).

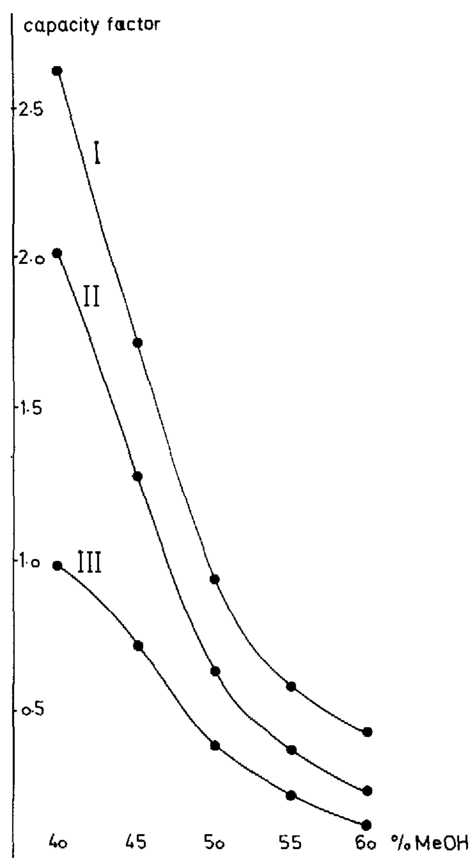

Fig. 2. Capacity factors of hymecromon, hymecromon-5-O-4-β-D-glucuronide and hymecromon-7-O-sulphate in different solvent compositions.

Analysis was performed isocratically with a mobile phase consisting of aqueous 0.01 *M* tetrabutylammonium bromide pH 4.7 and methanol (60:40, v/v). The mobile phase was prepared daily, filtered through a Millipore 5-μm filter, and degassed by sonification. The column was conditioned with 150 ml of the mobile phase, and run overnight with the same, using a flow-rate of 0.1 ml min^{-1}. During analysis the flow-rate was maintained at 1.8 ml min^{-1}.

RESULTS AND DISCUSSION

Ion-pair reversed-phase HPLC using an isocratic solvent composition was found to provide a reliable method to separate the compounds studied. Solvent compositions between 40% and 60% methanol, using the liquid chromatographic conditions mentioned above, resulted in the clean separation of hymecromon from its glucuronide and sulphate conjugates (Fig. 2). The system containing 40% methanol is useful in estimating the concentrations of the drug and its conjugates in biological fluids, in pharmacokinetic studies, and in clinical situations without interference from endogenous materials (Fig. 3).

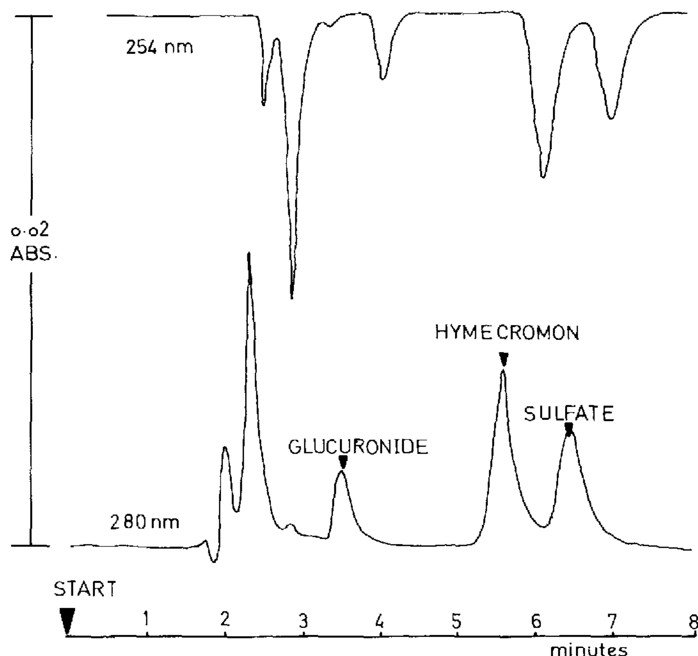

Fig. 3. Liquid chromatogram from plasma containing 100 mg l^{-1} hymecromon, 50 mg l^{-1} glucuronide, and 75 mg l^{-1} sulphate.

Quantitation

Standard curves for compounds I—III in plasma have been prepared by analyzing standard plasma concentrations according to the method described above. Peak areas were plotted against concentrations, and linear regression analysis was performed on the data.

Recovery

For serum containing 10 mg l^{-1} the total recovery was 87.6 ± 1.4%.

Reproducibility

The reproducibility of the assay was convenient. For fifteen independent determinations over a period of three weeks, the coefficient of variation was less than 7.5% for 5—40 mg l^{-1}. The intra-assay coefficient of variation measured from replicate analyses of standard solutions prepared in serum containing hymecromon at concentrations of 2.0, 10.0 and 20.0 mg l^{-1} were 2.8%, 2.5% and 2.1%, respectively.

Limit of sensitivity

The limit of accurate measurement of the assay was 0.2 mg l^{-1}; the intra-assay coefficient of variation at this concentration was 7.5%. This is adequate when it is considered that the average plasma concentration in specimens obtained from patients receiving 200 mg of this compound intravenously is about 5—40 mg l^{-1}.

Application

A typical plasma concentration profile of compounds I—III in man is shown in Fig. 4. Detailed pharmacokinetic analysis on normal subjects, and those suffering from various well-defined hepatic diseases will be published elsewhere.

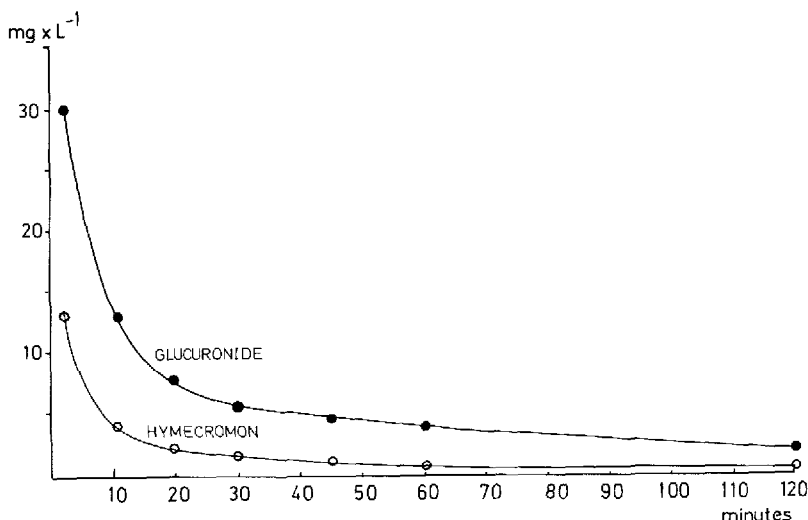

Fig. 4. Plasma concentration profile of hymecromon and the glucuronide conjugate in a human subject following intravenous administration of 200 mg of hymecromon.

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