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Short Communication

Simple high-performance liquid chromatographic method for the determination of a new phytochemical drug, fellavine, and its metabolites in human and rat plasma and urine

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ABSTRACT

The use of a small precolumn instead of an injection loop for the determination of a new phytochemical drug, fellavine, and its metabolites is described. The method combines the direct injection of plasma and urine into the reversed-phase precolumn with separation on a Spheri-5 RP-18 analytical column. Different sorbents in the precolumn were compared. A recovery of fellavine and its metabolites from biological fluids except rat plasma of almost 100% was achieved on Chrompack RP (30–40 μm) and LiChrosorb RP-18 (7 μm). For rat plasma only the last sorbent gave 80% fellavine recovery. The influence of the protein binding on the fellavine recovery was examined. The limit of detection was equal to 0.05 μg/ml fellavine for plasma and 0.02 μg/ml for urine. To enhance the limit of detection longer precolumns were preferred.

INTRODUCTION

For the fast high-performance liquid chromatographic (HPLC) separation and quantification of drugs and their metabolites from biological fluids the direct column injection technique may be used. The main disadvantage of this method is the reduction in column life. Therefore methods based on the direct injection of a biological fluid into a small column instead of the injection loop were developed [1–3]. Such precolumns have been used for trace enrichment procedures, as well as for sample clean-up. Using this technique it is also possible to achieve almost 100% recovery of a drug and its metabolites simultaneously [3].

In this communication an analysis method with the application of the direct

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precolumn injection of a new phytochemical drug, fellavine [8-(3-methylbut-2-enyl)-5,4′-dihydroxy-7-O-β-D-glucopyranosylflavanonol, Fig. 1], and its metabolites in human and rat plasma and urine is proposed; the method was developed for subsequent pharmacokinetic study. In the case of fellavine this method significantly improves the peak form of the drug compared with the traditional extraction and deproteinization procedures.

EXPERIMENTAL

Chemicals

Fellavine was supplied by the Institute of Plant Substances (Moscow, USSR). Acetonitrile was HPLC grade (Reachim, Charkov, USSR). Acetic acid (chemical grade) (Reachim, Charkov, USSR) was frozen, and the liquid fraction was eliminated. The remainder after thawing was distilled and the fraction with the boiling point of 116.5–117.5°C was used. Glass-distilled water was used throughout.

Equipment

The pump was a Gilson 302 with a 5SC pump head (Gilson, Villiers-le-Bel, France). The sample injector was a Rheodyne syringe-loading injector (Model 7125; Rheodyne, Berkeley, CA, USA). Two kinds of precolumns were used. Precolumn 1 (75 mm × 2.1 mm I.D., Chrompack, Middelburg, Netherlands) was dry-filled with the Chrompack RP sorbent (particle size 30–40 μm). Precolumn 2 (20 mm × 2 mm I.D., direct connect refillable guard column, Alltech Assoc., Deerfield, IL, USA) was dry-filled with different sorbents (Chrompack RP, LiChrosorb RP-18, particle size 7 μm, Merck, Darmstadt, Germany; a C_{18} pellicular packing, Alltech Assoc.; and Silasorb C_{18}, particle size 15 μm, Chemapol, Prague, Czechoslovakia). The precolumns replaced the sample loop in the injection valve. The analytical column Spheri-5 RP-18 (particle size 5 μm, 100 mm × 2.1 mm I.D.; Brownlee Labs., Santa Clara, CA, USA) was equipped with a Spheri-5 RP-18 guard column (particle size 5 μm, 30 mm × 2.1 mm I.D.). Detection was performed with a 112 UV–VIS absorbance detector (Gilson) at 280 nm.

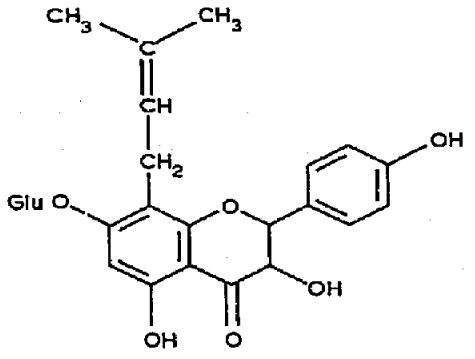

Fig. 1. Chemical structure of fellavine.

Analytical mobile phase

The mobile phase (acetonitrile–1% acetic acid; 26:74, v/v) flow-rate was 150 μl/min. The water and the mobile phase were passed through a 0.45-μm filter (Millipore, Bedford, MA, USA) and degassed *in vacuo* prior to use.

Precolumn purification and concentration

The extraction precolumn was purged on the load position of the injection valve with five column volumes of water to waste (1 ml for precolumn 1 and 0.3 ml for precolumn 2). The samples were centrifuged (8800 *g*, 2 min) and loaded on the precolumn. The impurities were washed out with another five precolumn volumes of water.

The samples were injected by back-flushing them with the chromatographic eluent into the analytical column and the precolumn remained in the "inject" position while the analysis was completed.

RESULTS AND DISCUSSION

Traditional methods of plasma and urine extraction and deproteinization with three volumes of acetonitrile or methanol [4] in the case of fellavine caused deterioration of the peak shape, because the final organic solvent concentration in the extracts was approximately 75% compared with 26% in the mobile phase. The evaporation of the extracts and their subsequent dilution with the mobile phase greatly increased the time of the analysis.

Using the direct precolumn injection allowed us to avoid these drawbacks (Fig. 2) and in addition allowed the simultaneous analysis of fellavine and its three metabolites in urine (Fig. 3).

Packing material

The main problem of the direct injection technique is the selection of the extraction precolumn sorbent. It has been shown that the smaller the sorbent particle size (and the more the surface), the greater the drug recovery but the less the extraction precolumn and analytical column lifetime. Also the less the drug polarity, the greater sorbent particle size may be used [5,6]. The sorbent in the precolumn was changed when 10% fellavine peak height reduction was observed. We could inject a total of up to 16 ml of plasma into precolumn 1 and up to 0.3 ml of plasma into precolumn 2 (filled with LiChrosorb RP-18, 7 μm) before the precolumns were damaged. The analytical column efficiency was the same during the entire work.

Precolumn enrichment

The limit of detection of the method was determined from the sample volume injected, however this parameter was limited by the precolumn capacity. The precolumn capacity depends on its dimensions, as well as on the type of the

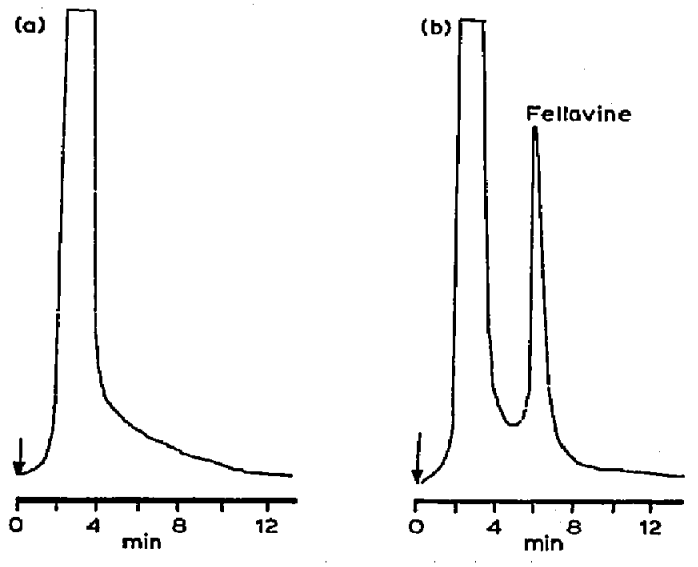

Fig. 2. Chromatograms for the separation of fellavine in human plasma: (a) 10 μl of blank plasma; (b) 10 μl of blank plasma spiked with fellavine (5 mg/l). Loop: precolumn 2 (20 mm × 2 mm I.D. packed with LiChrosorb RP-18, 7 μm). Eluent: acetonitrile–1% acetic acid (26:74, v/v). Flow-rate: 150 μl/min. Analytical column: Spheri-5 RP-18 (100 mm × 2.1 mm I.D.). Guard column: Spheri-5 RP-18 (30 mm × 2.1 mm I.D.). Column temperature: 20°C. Detection: UV at 280 nm, 0.02 a.u.f.s.

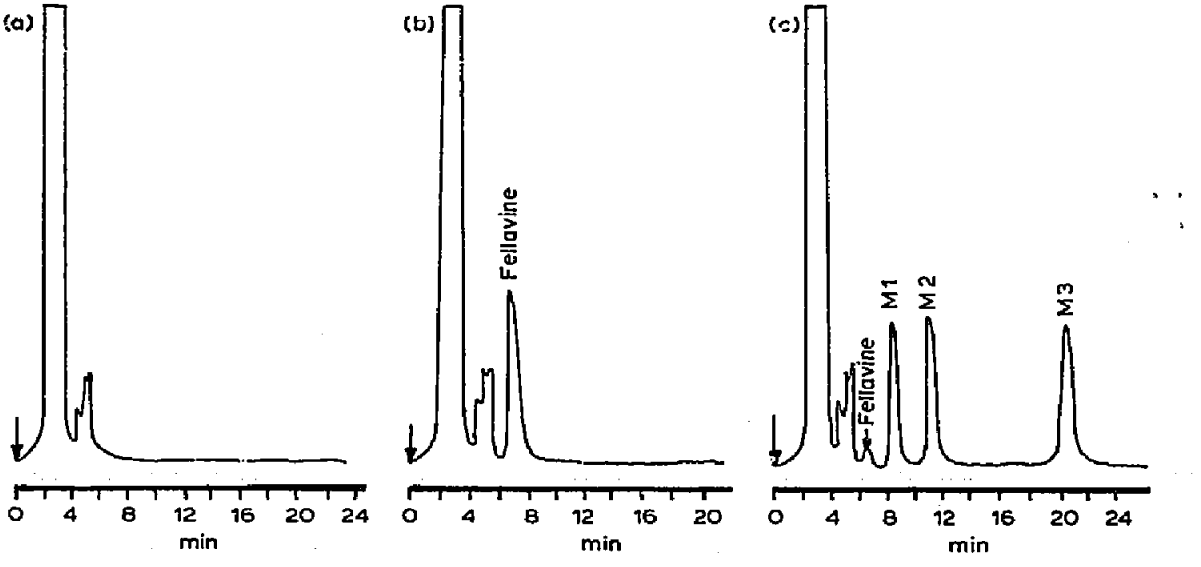

Fig. 3. Chromatograms for the separation of fellavine and its metabolites M1, M2 and M3 in human urine: (a) 10 μl of blank urine; (b)10 μl of urine spiked with the fellavine standard solution (2 mg/l); (c) 10 μl of urine 2–4 h after the oral fellavine administration (600 mg). Loop: precolumn 2 (20 mm × 2 mm I.D. packed with LiChrosorb RP-18, 7 μm). Eluent: acetonitrile–1% acetic acid (26:74, v/v). Flow-rate: 150 μl/min. Analytical column: Spheri-5 RP-18 (100 mm × 2.1 mm I.D.). Guard column: Spheri-5 RP-18 (30 mm × 2.1 mm I.D.). Column temperature: 20°C. Detection: UV at 280 nm, 0.02 a.u.f.s.

sorbent and on the particle size. The sample volumes were increased until the fellavine peak height plateau was observed. For precolumns 1 and 2 the maximum sample volumes at which linearity was maintained were 80 and 20 μl, respectively. These volumes were approximately proportional to the precolumn length. Therefore, to attain better limit of detection, longer precolumns should be used.

Washing fluid

Since the peak of fellavine is located close to the first peaks of endogenous serum and urine substances, most attention was given to the washing out of these impurities.

The influence of the washing fluid volume and its composition on drug recovery and elimination of impurities was investigated. It was shown that increasing the washing water volume to only 7–8 extraction precolumn volumes caused a reduction in the fellavine peak height. The best cleaning of the impurities was observed in the case of 1% acetonitrile, however, the drug recovery was only 70%.

Binding studies

The impact of the protein binding on the fellavine recovery was examined. This was achieved comparing the peak heights for the same concentrations of fellavine in water and plasma [4]. For human plasma identical recoveries (almost 100%) were obtained in both cases. For rat plasma the recoveries were 20 and 80% for Chrompack RP (30–40 μm) and LiChrosorb RP-18 (7 μm), respectively, assuming that protein binding influences fellavine adsorption.

Precision, linearity and sensitivity

For the determination of fellavine, an external standard was used. The calibration curves pass through the origin of the coordinates and are linear in the 0.02–20 μg/ml concentration range. For the calibration curve of fellavine in plasma and urine we obtained coefficients of variation of 1.3 and 1.2% (within-day) and 2.8 and 3.2% (day-to-day) (for ten injections). The limit of detection was 0.05 μg/ml for plasma and 0.02 μg/ml for urine.

CONCLUSIONS

Replacing the sample loop by the precolumn provides as rapid, routine determination of drugs, avoiding traditional time-consuming sample preparation steps. The method is convenient for the simultaneous analysis of drugs and metabolites in biological fluids and is especially suitable if drug extraction by traditional methods causes deterioration of the peak shape.

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