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ON-LINE SCREENING FOR DRUG METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A DIODE ARRAY UV DETECTOR

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SUMMARY

Rapid screening for and selection of potential metabolites of urapidil in dog urine by high-performance liquid chromatographic analysis is achieved by use of a diode array UV detector, which allows multi-wavelength detection and collects several complete UV spectra from each peak in the course of a single chromatographic analysis. Because of the similarity of metabolite spectra to the spectrum of the parent compound, few interesting peaks are picked out, rather rapidly, by means of a selection criterion based on absorption ratios. Inspection of the complete UV spectra of these pre-selected peaks allows further restriction to "candidate" metabolite peaks. Comparison of up-slope, apex and down-slope spectra is a convenient way of testing for peak purity.

INTRODUCTION

The search for and identification of unknown metabolites in biological fluids such as serum, blood and bile by high-performance liquid chromatography (HPLC) often represents a difficult task because of the complexity of the biological matrix, usually giving rise to a large number of peaks from which potential metabolites must be distinguished. Recognition of metabolites is especially difficult if they are present in low concentrations and if the composition of the matrix is variable. The usual method of solving this problem is to scan UV spectra of candidate peaks, after interrupting the solvent flow. This procedure may be repeated only once or twice. Hence, if a large number of peaks must be screened, many chromatographic analyses must be performed, and this often poses problems due to restricted sample amount.

A new way of obtaining as much UV information as possible from a single chromatographic analysis is to monitor the eluent with a diode array detector, which records several complete UV spectra of each peak, as it leaves the column. As drug metabolites often exhibit UV spectra that are quite similar to the parent compound,

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rapid screening of all peaks in a chromatogram represents a very useful means of finding potential metabolites, which may then be collected and investigated by mass spectrometry (MS). As the diode array detector used in this study takes several UV spectra of each eluted compound, the purity of the peaks of interest may also be tested by comparison of, *e.g.*, up-slope and down-slope spectra.

The application of this technique is demonstrated with urapidil[2] (Fig. 1), a new antihypertensive drug, which is extensively metabolized in animals[3] and therefore a good example for demonstrating the advantages of multi-wavelength detection. In addition to the rapid recognition of potential metabolites of urapidil, the peak purity of urapidil itself was checked and found to be inadequate. As a consequence, the resolution of the analytical system had to be improved.

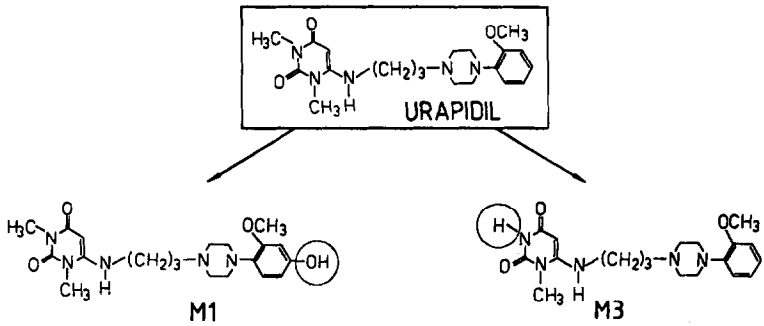

Fig. 1. Hitherto known metabolic pathway of urapidil in the dog. Urapidil breaks down to metabolite M1 by *p*-hydroxylation, to metabolite M3 by nitrogen-dealkylation.

EXPERIMENTAL

The analytical system consisted of a HP 1084B liquid chromatograph, the outlet of which was connected to a HP 1040A diode array detector.

The columns used were from Bischoff (Leonberg, F.R.G.), and were filled with Nucleosil RP-18, 5 μm. The mobile phase was a mixture of 20 m*M* aqueous sodium perchlorate (adjusted to pH 2.0 with perchloric acid) and of methanol. The analyses were performed at room temperature; the injection volume was 5 μl.

The biological sample consisted of dog urine (0–8 h), which was collected after a single oral administration of 10 mg/kg urapidil. For analysis, part of the fraction was diluted (1:1, v/v) in mobile phase.

RESULTS

Fig. 2 shows a chromatogram, obtained after injection of 5 μl of diluted dog urine, containing urapidil and several metabolites of urapidil. The chromatogram was recorded simultaneously at six different wavelengths from which two (230 and 268 nm) were selected. Besides the urapidil peak, there are two metabolite peaks (M1 and M3) with known structures (Fig. 1). As the urapidil UV spectrum shows a maximum at 268 nm and a minimum at about 230 nm (Fig. 3), a useful selection criterion

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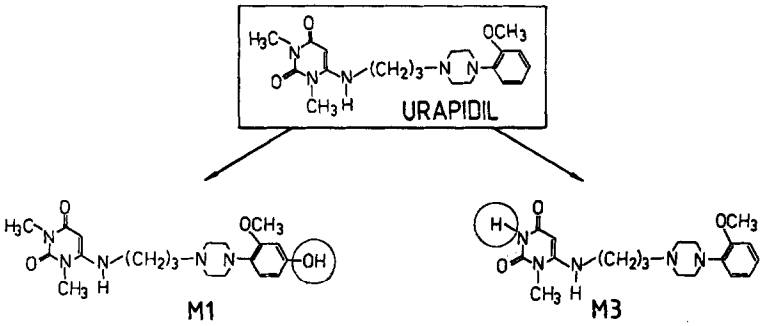

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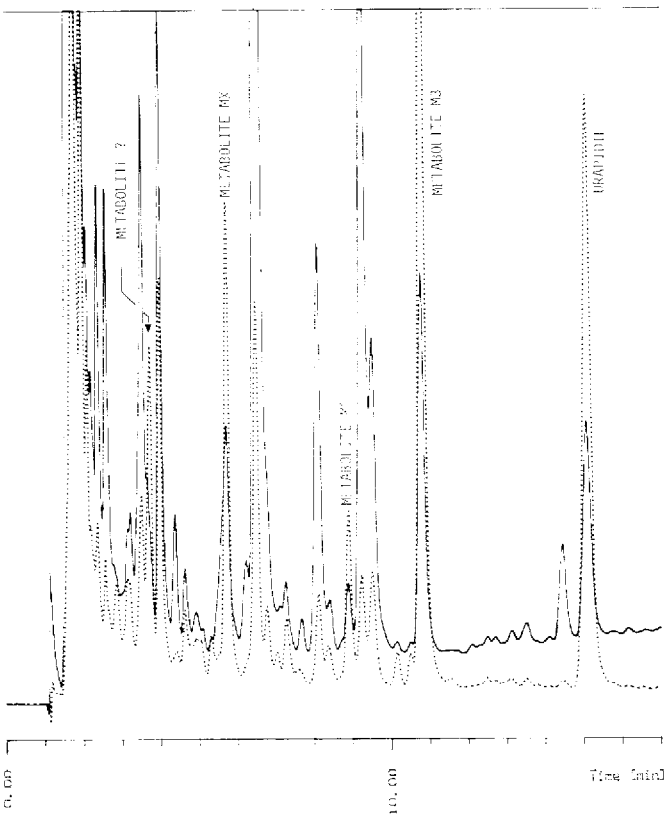

Fig. 2. HPLC chromatogram of dog urine (0–8 h fraction) after a single oral administration of 10 mg urapidil per kg body weight. Chromatograms were plotted at 230 nm (—) and 268 nm (····) (each 4 nm bandwidth), with 550 nm (100 nm bandwidth) as reference. Chromatographic conditions: column, 125 × 4.6 mm I.D., Nucleosil®, RP-18, 5 μm; flow-rate, 1.0 ml/min; temperature, ambient; gradient, 20 m*M* sodium perchlorate (pH 2.0 with perchloric acid)–methanol; initial isocratic period, 2 min at 15% methanol; within 13 min to 30% methanol.

for the recognition of new unknown metabolites of urapidil appears to be the condition that UV absorption 268 nm > 230 nm.

Thus, whenever the dotted line in Fig. 2 is significantly higher than the full line, a metabolite is potentially present. This is the case with the (known) metabolites M1 and M3 (retention times 8.8 and 10.7 min) and with two further peaks at retention times of 5.7 and 2.7 min, denoted as metabolite Mx and "metabolite?", respectively. As the UV spectra of metabolites are usually quite similar to that of the parent compound, further evidence for new metabolites may be gained by comparison of the UV spectra of the unknown peaks with the UV spectrum of pure urapidil. As may be seen from Fig. 3, the apex UV spectrum of metabolite Mx shows the same characteristics as the UV spectrum of standard urapidil, whereas the apex spectrum of the peak at 3.7 min ("metabolite?") departs from it significantly. Thus, metabolite Mx should be further investigated by semipreparative isolation and subsequent structure elucidation by MS, whereas the peak at 3.7 min is most probably unrelated to urapidil.

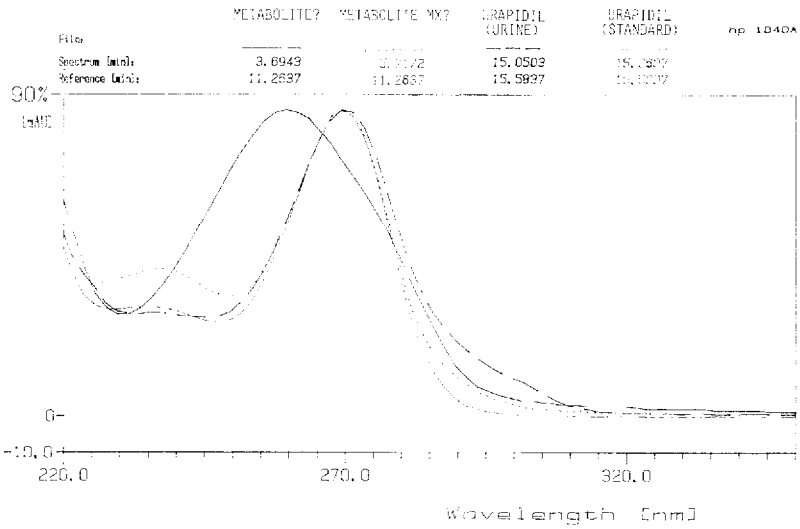

Fig. 3. UV spectra of three peaks (urapidil, metabolite Mx and "metabolite ?") in the HPLC chromatogram of the urine sample in comparison with an urapidil spectrum, recorded during chromatography of a standard solution.

Fig. 3 shows the apex UV spectrum of the urapidil peak in Fig. 2 in comparison with the UV spectrum of a standard urapidil peak. The departure of the urine urapidil peak spectrum from the standard spectrum clearly shows that the urapidil peak in Fig. 2 is impure and contains one or more further components, which prevent precise quantification of urapidil. As a consequence, the resolving power of the system was increased by changing to a 250-mm column. When the sample was chromatographed on this column the chromatogram in Fig. 4 was obtained. Comparison of Figs. 2 and 4 shows that an additional peak with a slightly longer retention time is separated from urapidil, although the background peak seen in Fig. 2 at 14.5 min is now closer to the urapidil peak in Fig. 4. The latter fact as well as the resolution of the apparently symmetrical "urapidil" peak in Fig. 2 into urapidil and a new background peak might be ascribed to a different selectivity of the 250-mm Nucleosil column (different batch?) as compared to the 125-mm Nucleosil column. An additional influence explaining the somewhat different peak pattern in Fig. 4 may have been the slightly different gradient used in Fig. 4.

The apex UV spectrum of the urapidil peak in Fig. 4 is shown in Fig. 5 and now exactly coincides with the UV spectrum of an urapidil standard. The UV spectrum of the background peak at the retention time of 15.0 min is also shown in Fig. 5 and exhibits a maximum and a shoulder at longer wavelengths than the absorption maximum of urapidil, which explains the departure of the spectrum of the impure urapidil peak in Fig. 2 from the spectrum of an urapidil standard in Fig. 3.

In Fig. 6 the UV spectrum of a standard of metabolite M3 is compared with the apex spectrum of M3 in urine, as separated in Fig. 4. The nearly complete coincidence of the two spectra proves the purity of the M3 peak in Fig. 4. Also in Fig. 6 are collected the apex UV spectra of the potential metabolites Mx and "metabolite?". Direct comparison reveals a striking similarity of the spectrum of Mx with

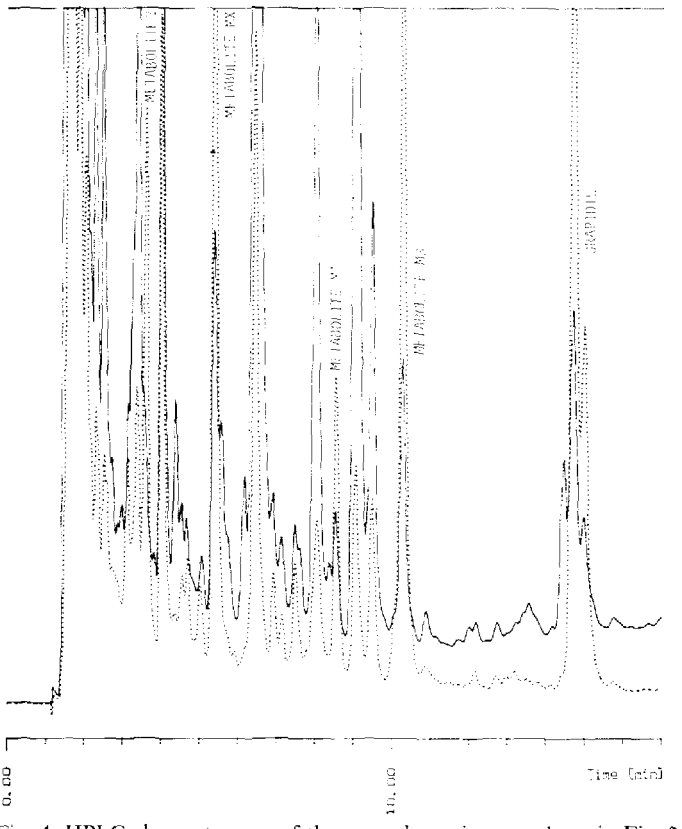

Fig. 4. HPLC chromatogram of the same dog urine sample as in Fig. 2. Column: 250 × 4.6 mm I.D. Flow-rate: 1.5 ml/min. Gradient: within 8 min from 15% to 25%, initial isocratic period 2 min at 15%. All other conditions as in Fig. 2.

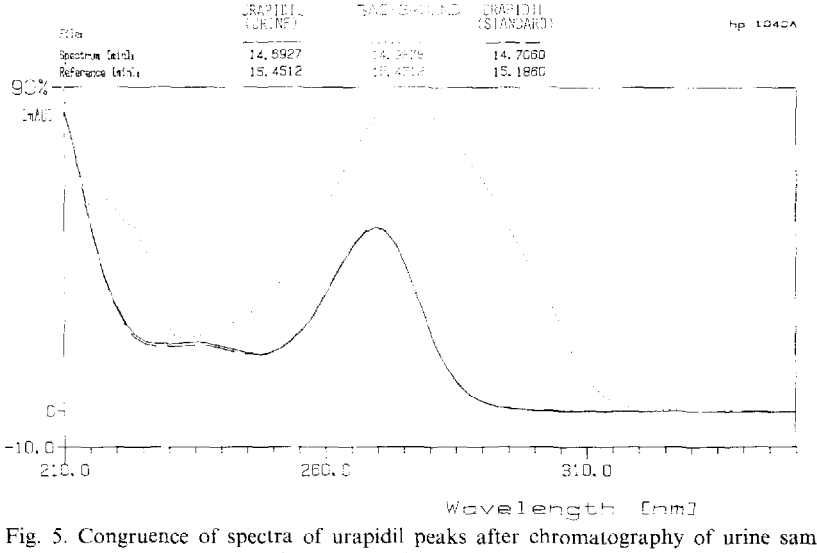

Fig. 5. Congruence of spectra of urapidil peaks after chromatography of urine sample and standard solution in comparison with the separated background material in the urapidil peak of the urine sample.

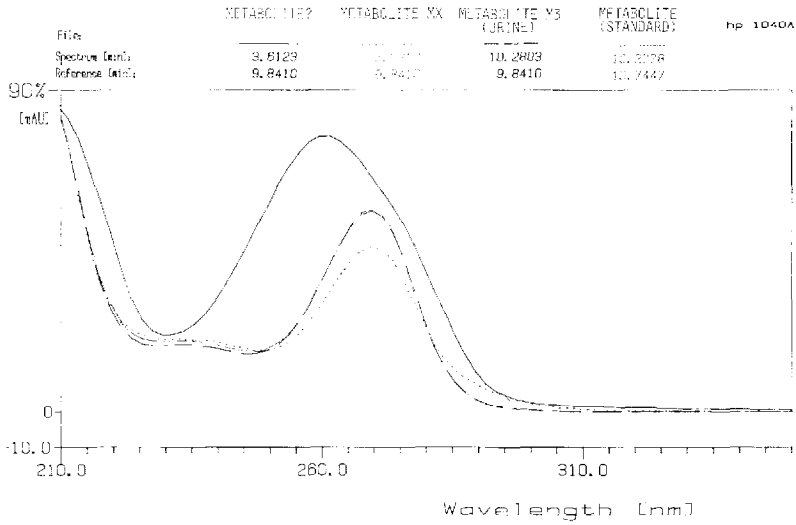

Fig. 6. Comparison of peak spectra of metabolite M3, Mx and "metabolite ?" with the spectrum of the standard peak.

the spectrum of M3, whereas the spectrum of "metabolite?" has a quite different characteristic. This peak therefore should not be selected for structure elucidation.

Finally, in Fig. 7 the UV spectra of the peaks of urapidil metabolites M1 and M3 and of metabolite Mx, obtained from the chromatogram in Fig. 4, are compared. Again there is a close relationship, underlining the usefulness of this concept in a rapid search for unknown metabolites.

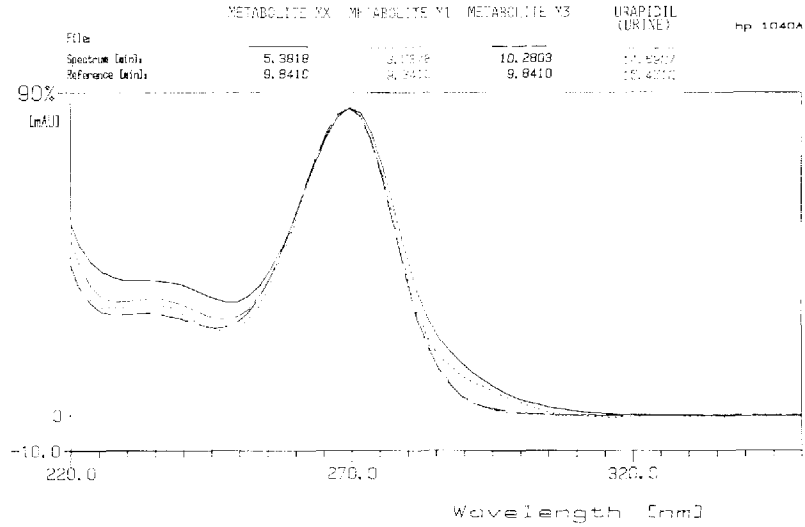

Fig. 7. UV spectra of peaks in the chromatogram of a urine sample. The congruence demonstrates the similarity of the urapidil spectrum with those of potential metabolites.

CONCLUSIONS

Multi-wavelength detection in HPLC analysis by means of a diode array UV detector greatly facilitates the search for unknown metabolites of drugs in biological samples, such as urine. By application of a suitable selection criterion (absorption at 268 nm > 230 nm), few interesting peaks can be rather rapidly distinguished from a forest of "background peaks" within a single chromatographic analysis, as was demonstrated with the potential metabolites Mx and "metabolite ?". Inspection of the complete UV spectra of the peaks of interest leads to a further selection of compounds with UV spectra similar to that of the parent compound, as was the case with metabolite Mx.

Another important finding was that peak symmetry does not necessarily imply peak purity, as was demonstrated with the parent compound urapidil.

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