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Automated screening for drug metabolites with high-performance liquid chromatography, UV-visible diode array detection and spectra library search*

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Introduction

High-performance liquid chromatography (HPLC) in combination with UV-visible diode array detection is widely used for the screening of drug metabolites in biological fluids [1, 2]. Zech *et al.* [1] reported on a method which used multi-wavelength detection and signal ratios as a criterion to preselect chromatographic peaks as metabolite candidates. Positive candidates have been further examined by a manual interactive comparison of the UV spectrum with the spectrum of the parent drug. The interactive comparison is time consuming and therefore unsuitable for large sample throughputs.

In the present paper a method is described in which spectra are compared automatically after each analysis. If a peak is found as a metabolite candidate, the match factor is printed at the peak apex. The search algorithm is described as well as some parameters influencing the reliability of the result.

The application of the technique is demonstrated with urapidil [3], an antihypertensive drug which is extensively metabolised in animals. The metabolic pathway of urapidil has been described previously [1].

Experimental

The experiments were carried out on a Hewlett-Packard 1084A Liquid Chromatograph. The outlet was connected to an HP 1040M diode array detector. For data

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evaluation, an HP 79994A ChemStation was used; this consists of an HP 9000 series 300 computer, a 20 Mbyte Winchester disc drive and a ThinkJet printer.

The 250 × 4.6 mm i.d. column (Bischoff, Leonberg, FRG) was filled with 5-μm Nucleosil RP-18. The mobile phase was a mixture of 20 mM aqueous sodium perchlorate (adjusted to pH 2.0 with perchloric acid) and of methanol.

The biological sample which consisted of human urine was collected after a single oral administration of 60 mg of urapidil to a healthy young subject.

During the chromatographic analysis, UV-visible spectra were recorded and stored on the Winchester disc drive during elution by using a peak detector. After the run, the spectrum of each individual peak was compared with the spectrum of the parent drug. If the match between the peak spectrum and the spectrum of the parent drug exceeded a specified value, the peak was assumed to be a metabolite of urapidil and the match factor was printed at the top of the peak.

Similarity between spectra of metabolites and the parent compound to a certain degree is a prerequisite for searching metabolites by this comparative technique. Breakdown to phase I or II metabolites, however, normally does not affect the chromophore of the parent compound to such an extent that the characteristic of the spectrum is lost completely. Therefore, the probability of very similar metabolite and parent compound spectra is very high.

To calculate the match between different UV-visible spectra, the least-square fit correlation coefficient through all corresponding absorbances, was used. The match factor indicates the similarity between two spectra. A match factor of 0 indicates that there is no match and 1000 indicates identical spectra.

Before the chromatographic analysis, some parameters were specified in the search program. These included the wavelength range, the mode of spectral background subtraction and the lower limit for the match factor. The wavelength range chosen was 250–300 nm. From experience, the similarity between the parent drug spectrum and the metabolite spectra is most significant within this range. As the lower limit for the match factor, 900 was specified.

Before the first sample analysis, a urapidil standard was analysed and the spectrum was stored together with the name of the compound and the retention time, in a library.

Results and Discussion

Figure 1 shows a chromatogram obtained after injection of 10 μl of human urine. Besides the parent drug, two peaks have been labelled as positive metabolite candidates with a match factor of 989 at retention time 15.8 min and with a factor of 978 at retention time 17.61 min. The amounts of the metabolites and the parent drug are in the lower nanogram range. The signal height is less than 10 mAU.

The urapidil peak was labelled as the parent drug because the retention time of that peak is within a specified window of the retention time of urapidil as stored in the library. Other peaks outside the window with a higher match as specified before the run are labelled as metabolites and with the calculated match factor.

Positive candidates have been further examined by a visual comparison. Figure 2 shows an overlay of the normalised spectra from the urapidil standard and the two metabolites. The comparison shows that all three spectra have a maximum at around 270 nm. However, from this comparison a similarity with a match of higher than 900 is not obvious when doing a point-to-point comparison over all wavelengths.

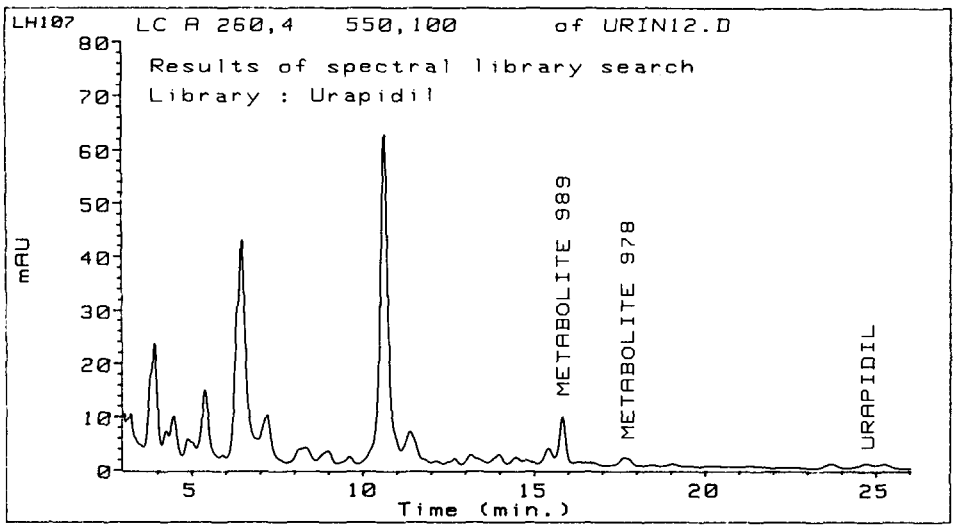

Figure 1
Result of automated screening for urapidil metabolites in human urine. Wavelength range: 250–300 nm, with baseline correction. Chromatographic conditions: 250 × 4.6 mm i.d. column packed with 5-μm Nucleosil; flow rate: 1.0 ml min^{-1}; gradient, 20 mM sodium perchlorate (pH 2.0 with perchloric acid)–methanol; initial isocratic period, 2 min at 15% (v/v) methanol; within 15 min to 30%, (v/v) methanol.

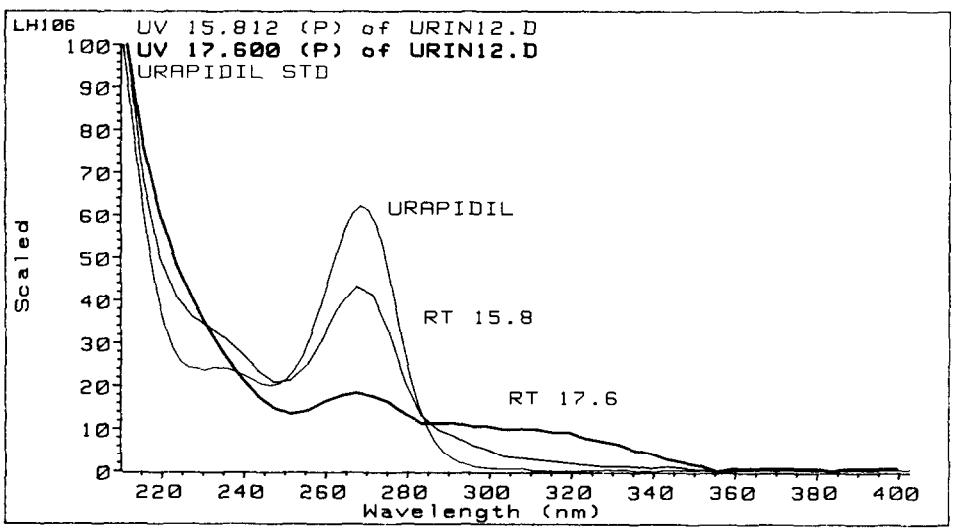

Figure 2
Raw UV spectra of urapidil standard and possible metabolite candidates.

The reason for the discrepancy is that the spectra in Fig. 2 are normalised to the highest absorbance which for all spectra is within the examined range at 210 nm. At very low sample amounts, the spectral contribution from the mobile phase in the lower UV range is significant. Therefore, for the library search a spectral background compensation was specified. Before a spectrum from the peak apex is searched within a library a

spectrum acquired at the baseline following the peak is subtracted from the apex spectrum. Figure 3 shows a comparison of normalised spectra of urapidil and of the metabolite candidate at retention time 15.8 min with a baseline correction. Now the similarity becomes obvious.

In the upper right window of Fig. 3 the metabolite spectrum is shown with the absolute absorbance scale. It shows that the search can be successfully used even with an absorbance range of less than 10 mAU.

If the amounts are even lower, there is a very high risk that wrong results are obtained

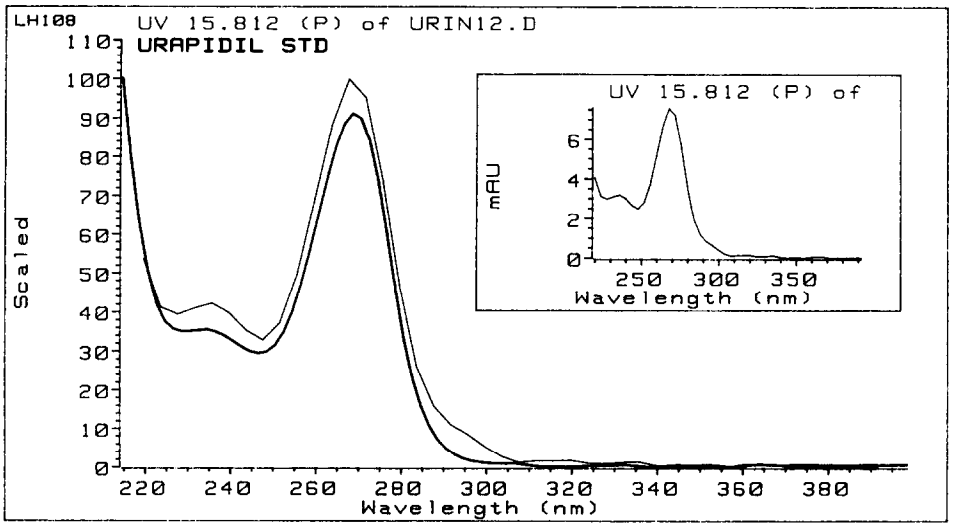

Figure 3
Baseline corrected spectra of urapidil and of the peak at retention time 15.7 min.

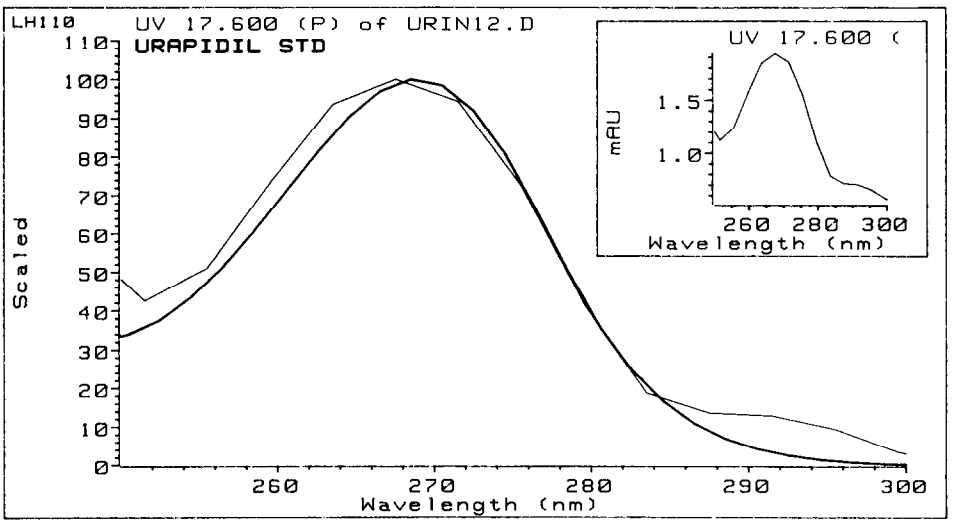

Figure 4
Spectra of urapidil and of the peak at retention time 17.6 min.

if the comparison is made over all wavelengths starting from 210 nm. Therefore, when the highest sensitivity was required, a wavelength range between 250 and 300 nm was chosen. Figure 4 shows a comparison of the urapidil spectrum and of the peak spectrum at retention time 17.6 min. A match factor of 978 was calculated for the two spectra.

Conclusions

It was demonstrated that human urine samples can be screened automatically for drug metabolites by comparing the spectrum of each individual peak with the spectrum of the parent drug, if the metabolite spectra are identical or similar to that of the parent drug. If the metabolites are expected to be detected in low concentrations, the spectra should be baseline compensated to eliminate the spectral contribution of the mobile phase. In addition it is recommended that a wavelength range is selected in which the highest spectral similarity between the metabolite and parent drug spectra is expected and the spectral contribution of the mobile phase is low.

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