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The use of ultraviolet spectra and chromatographic retention data as an aid to metabolite identification

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Abstract: Phenoxypropanolamines and their putative 4-hydroxy metabolites have been chromatographed using reversed-phase HPLC and UV spectra recorded using a linear diode array detector. Analysis of the chromatographic data using the functional group contribution approach allowed peaks to be tentatively characterized as hydroxy metabolites. This characterization was further enhanced by the examination of the UV spectra which showed distinct changes following hydroxylation. Used in combination, the two techniques allow discrimination between the possible isomeric hydroxy metabolites. The chromatographic and spectral changes observed with the relatively complex phenoxypropanolamines can be successfully modelled using simple compounds to further facilitate identification.

Keywords: *High-performance liquid chromatography; reversed-phase; functional group contribution approach; ultraviolet spectra; bathochromic shift; phenoxypropanolamine; metabolite; hydroxylation.*

Introduction

Metabolite identification can play an important role in drug discovery and drug development. The classical metabolite identification procedures involving mass spectrometry and proton resonance spectroscopy can require extensive sample clean up and purification prior to the identification proper. This approach, which may involve many weeks or months of work is generally unacceptable in the early phase of drug development, where a rapid response is essential in order to assist the discovery program.

More recently combined liquid chromatography–mass spectrometry (LC–MS) has been employed which avoids much of the lengthy sample clean up work. In certain instances however, this does not give the necessary structural information required for an unequivocal identification, particularly with regioisomers. This approach can also be very costly, and for optimal use requires a high level of technical input and support.

There are however, a number of alternative approaches that could be used, based on readily accessible techniques and instrumentation. In this respect the combined use of the linear diode array (LDA) ultraviolet (UV) detector with the functional group contribution approach to HPLC data analysis could give valuable information rapidly, and with the minimum of cost.

The analysis of chromatographic data using the functional group contribution approach has been put forward by a number of authors as a simple means of identifying metabolites [1–3]. Although these data can be obtained relatively quickly the 'identification' is far from unequivocal, due to the low information content of UV spectra. This may be one reason why this approach appears to have seen little use.

The LDA detector is now widely used in the area of liquid chromatography where the facility to carry out spectral suppression, spectral deconvolution and the generation of absorbance ratios, often in real time, have proved particularly useful [see ref. 4]. In basic operation the LDA allows UV spectra to be obtained relatively simply, without the necessity of extensive clean up which was previously essential. Since many metabolites show distinct differences in their UV spectra, compared to parent compound, the use of such spectra as an aid to metabolite identification would seem a logical application of the technique.

The use of the two techniques in combination, would appear to offer a powerful, yet simple approach to drug identification. The present work is aimed at demonstrating the utility of this combined approach through the characterization of putative 4-hydroxy metab-

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olites of a range of phenoxypropanolamines. These hydroxy derivatives were chosen as being representative of a common metabolic transformation.

Experimental

Apparatus

HPLC was performed using a 100 × 4.8 mm i.d. column packed with Hypersil 5 μm ODS (Shandon Southern Products Ltd., Runcorn, UK). Eluent was delivered using a Waters 600 Multi-solvent delivery system and injection was made via a Rheodyne 7125 valve. The eluent was monitored and UV spectra (210–400 nm) were recorded using a Hewlett–Packard 1040M LDA detector interfaced to a Hewlett–Packard 300 series computer.

Materials

Acetonitrile was HPLC grade from Fisons (Loughborough, UK) hexylamine (99%) was obtained from Aldrich (Gillingham, UK) and phosphoric acid (85%) was from BDH (Liverpool, UK). Anisole (methoxy benzene), 2-hydroxy, 3-hydroxy and 4-hydroxyanisole were obtained from Aldrich and used as received. The remainder of the solutes were obtained from the ICI compound collection. All solutes were dissolved in water at *ca* 1 mg ml^{-1} with the addition of dilute hydrochloric acid or acetonitrile to facilitate dissolution where necessary.

The HPLC eluent for the bulk of the work was acetonitrile–water–hexylamine–phosphoric acid (85%), (300:700:1.4:1.0, v/v/v/v), pH 2.7, delivered at a flow rate of 1 ml min^{-1}. To allow for variations in the lipophilicity of some of the model compounds the eluent acetonitrile content was adjusted accordingly.

The major compounds studied possessed the core phenoxypropanolamine or naphthoxypropanolamine structures shown in Fig. 1. Initially 11 pairs of compounds were studied, each pair differing in the presence of a 4-OH function on the core structure. These compounds possessed a wide range of terminating groups (R) including amides, esters, morpholines or carboxylic acids (Fig. 2). The basic pK_a values ranged from 7.7 to 9.5 and log *P* from 0.03 to 5.7.

Method

All samples were chromatographed at least twice to give retention values within 2%. The system dead time (t_o) was determined by the injection of lithium nitrate in water (10 μl of 25 mg ml^{-1}).

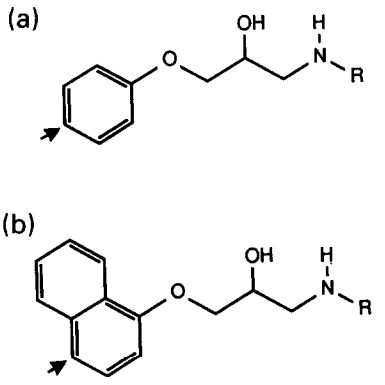

Figure 1
The phenoxy (a) and naphthoxypropanolamine (b) core structures (X) showing the site of hydroxylation (➔).

Retention data are reported as capacity factors (k') given by the relationship

$$k' = (t_r - t_o)/t_o,$$

where t_r is the solute retention time. The relationship between the retention of the parent and hydroxy compounds was quantified using the functional group contribution value (τ), where

$$\tau = \log (k'_d/k'_p)$$

and k'_p and k'_d are the capacity factors of the parent and hydroxy derivative, respectively.

Absorption λ_{max} values were measured and the bathochromic shifts (Δ) for the bands at low (*ca* 220 nm) and high (270 nm) wavelength were calculated.

Results and Discussion

Group contribution analysis

A typical HPLC chromatogram for one pair of phenoxypropanolamines (II/IIa) is shown in Fig. 3 and the retention data for all the compounds are given in Table 1. The tabular data are arranged as compound pairs, each differing only in the presence of the 4-hydroxy function. As expected, the more polar hydroxy analogues eluted prior to the parent compound in the reversed-phase system, resulting in negative functional group contribution values (τ).

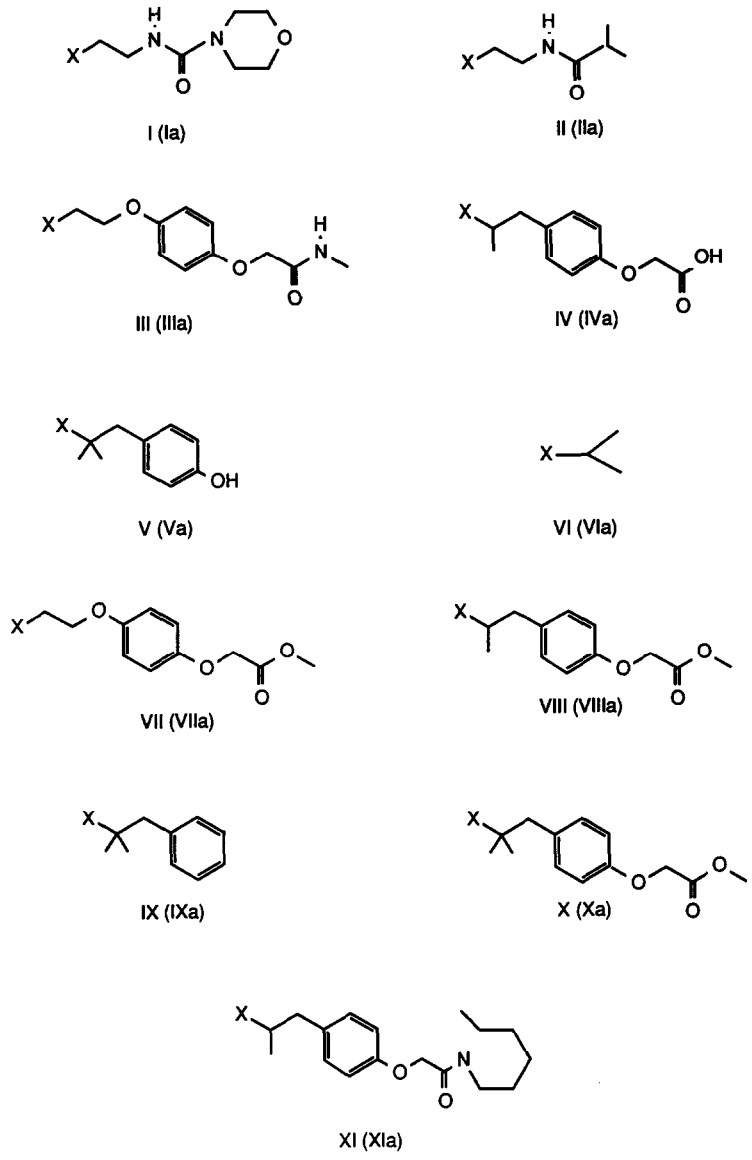

Figure 2
The substituent groups (R) attached to the core structure (X) shown in Fig. 1. The code number of the parent compound is given together with the number of the 4-hydroxy analogue in parenthesis.

Examination of the τ values for the 11 pairs of compounds (Table 1) shows them to be remarkably consistent, with a mean and standard deviation of -0.59 ± 0.030 (RSD 4.4%). Thus, despite the wide variation in the structures of the molecules studied (i.e. amides, esters, acids, etc.), 4-hydroxylation produces a very characteristic and reproducible shift in retention.

It is obvious therefore that the group contribution method could be used to aid metabolite identification. Calculation of τ for a pair of related HPLC peaks (i.e. parent compound and putative metabolite) could allow assessment of the likelihood that the unknown peak was actually a 4-hydroxy metabolite.

Although τ values are particularly useful in confirming that a peak is not a 4-hydroxy-metabolite of a particular drug, the procedure does not provide unequivocal identification. Other metabolites and endogenous compounds could have similar retention and hence be mistakenly identified as 4-hydroxy compounds. To add further weight to the group contribution data, the UV spectra can be examined for changes indicative of 4-hydroxylation.

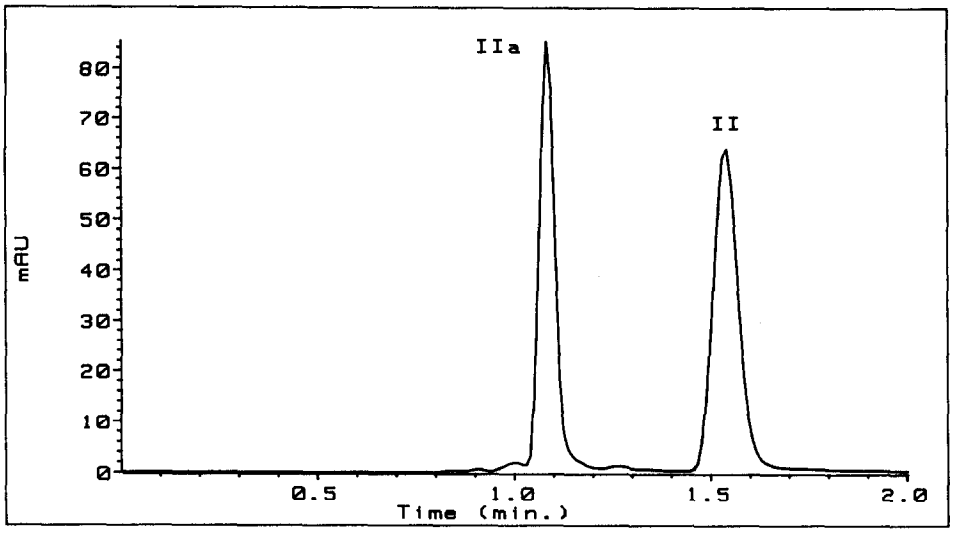

Figure 3
Typical HPLC chromatogram showing peaks for II and its 4-hydroxy metabolite (IIa).

Table 1
HPLC retention data (k') and group contribution values (τ) for 11 pairs of compounds, each pair differing only in the presence of a 4-hydroxy function

Parent compound	k'	4-Hydroxy derivative	k'	τ
I	0.34	Ia	0.09	−0.59
II	0.66	IIa	0.17	−0.58
III	0.84	IIIa	0.20	−0.63
IV	1.12	IVa	0.29	−0.58
V	1.82	Va	0.41	−0.64
VI	2.55	VIa	0.66	−0.59
VII	2.97	VIIa	0.78	−0.58
VIII	3.67	VIIIa	1.00	−0.57
IX	5.82	IXa	1.61	−0.56
X	6.42	Xa	1.49	−0.63
XI	29.74	XIa	8.61	−0.54

For experimental details see text.

Ultraviolet spectral analysis

The UV spectra for one pair of compounds is shown in Fig. 4. The phenolic metabolite (IIa) shows a distinct bathochromic shift for both the low (λ_1) and high (λ_2) wavelength bands, in comparison with the parent compound (II). The spectral shift is consistent with that for substitution of a hydroxyl group into an aromatic ring [5]. This effect was common to all the other compounds studied, and is clearly shown by the data in Table 2 which lists λ_{max} values and spectral shifts for the 11 pairs of compounds studied.

The fine structure, observable as shoulders on the sides of the band at *ca* 270 nm in the parent compound was usually lost on 4-hydroxylation to give a smoother more rounded spectrum for the derivative, typical of phenols (Fig. 4). Those compounds which retained these features on hydroxylation, either possessed an additional single phenoxy group in the substituent R (IVa Va, VIIa, Xa and XIa), or a naphthalene in the core structure (X).

It is clear that 4-hydroxy derivatives of phenoxypropanolamines (and naphthoxypropanolamines) have UV spectra which are distinctly different to those of the parent compound, even when the latter are *O*-alkyl substituted. The data would also suggest that this changes can be usefully employed as an aid to metabolite identification.

Isomeric hydroxy compounds

The above work was confined to 4-hydroxylation of an aromatic ring because of the commonplace nature of this chemical change in mammalian metabolism [e.g. see ref. 6]. How-

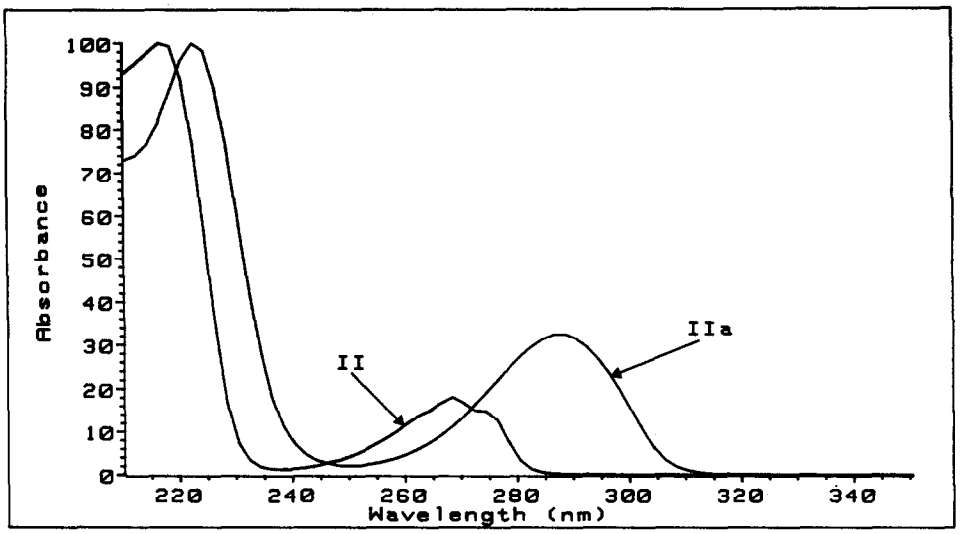

Figure 4
Typical UV spectra for a parent phenoxypropanolamine II and its 4-hydroxy metabolite (IIa).

Table 2
UV spectral characteristics (λ_{max1}, λ_{max2}, Δ_1 and Δ_2) for 11 pairs of compound, with each pair differing only in the presence of a 4-hydroxy function

Parent compound			4-Hydroxy derivative				
Compound	λ_1	λ_2*	Compound	λ_1	λ_2	Δ_1†	Δ_2†
I	<210	269	Ia	233	287	NC	18
II	218	269	IIa	223	287	5	18
III	221	277	IIIa	223	286	2	9
IV	222	268	IVa	224	280‡	2	12
V	222	275	Va	226	282‡	4	7
VI	215, 230s	292	VIa	<210, 243	317‡	NC	25
VII	222	278	VIIa	224	287	2	9
VIII	222	268	VIIIa	224	280‡	2	12
IX	<210	268	IXa	<210	288	NC	20
X	222	268	Xa	225	281‡	3	13
XI	222	268	XIa	224	280‡	2	12

s = Shoulder.
NC = not calculated.
*Fine structure present in all spectra.
†$\Delta_1 = \lambda_1$ derivative $-\lambda_1$ parent, $\Delta_2 = \lambda_2$ derivative $- \lambda_2$ parent.
‡Some fine structure present.

ever, although uncommon, hydroxylation can also occur at the 2-, or 3-positions in such rings or at other aromatic or non-aromatic sites within a drug molecule [6]. For example, hydroxylation of the *N*-hexyl side chain in XI would not be unexpected.

The combined approach of functional group contribution analysis and UV spectral interpretation would appear to be able to accommodate these variations in metabolic processes. This is shown in the study of several additional compounds.

In the case of compounds XII/XIIa (epanolol) the hydroxy group of XIIa occurs in the 4-position of a phenyl, as opposed to a phenoxy ring (Fig. 5). As expected, the τ value (−0.52) was similar to those for the compounds hydroxylated in the phenoxy ring. The spectral change however was quite different, both in terms of the wavelength shifts and the overall shape of the spectra compared to those observed previously (Fig. 4).

In a separate experiment a series of three isomeric ring hydroxylated derivatives of the

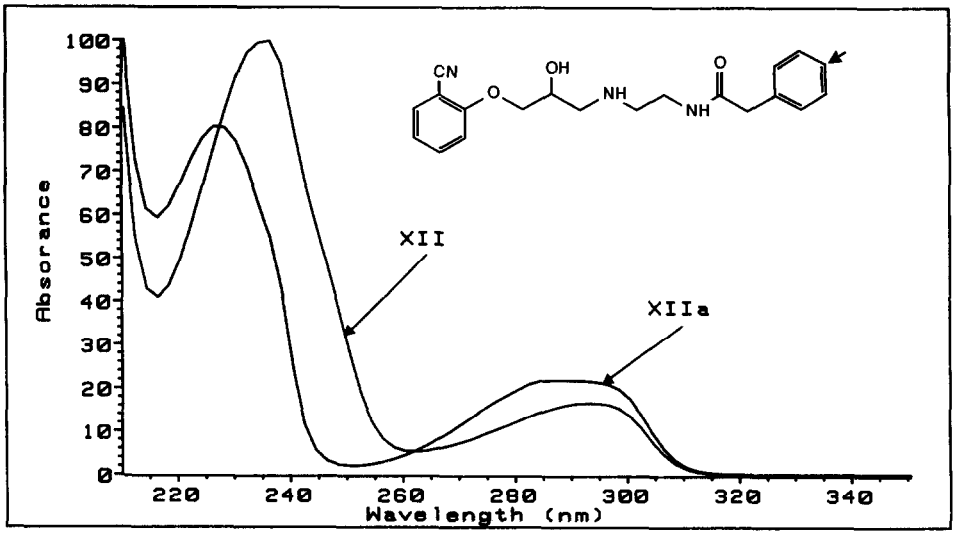

Figure 5
UV spectra and structures of XII and its 4-hydroxy phenyl analogue (XIIa, epanolol). The site of hydroxylation is indicated (➔).

beta-blocker 1-(isopropylamino)-3-(phenyloxy)-2-propanol were chromatographed. Because of the polar nature of these compounds this work was carried out using a slightly modified HPLC eluent, with the acetonitrile concentration reduced to 15%. Furthermore, because of the known variability of τ with organic modifier concentration [7] no attempt was made to relate the τ of these compounds with those discussed earlier. The ordering of elution was 4-OH < 3-OH < 2-OH < parent compound, with the k' values being; 0.12, 0.17, 0.29 and 0.54, respectively.

The hydroxy derivatives are clearly more polar than the parent compound, as noted above, and the difference in retention between the 2- and 4-hydroxy compounds can be explained by intramolecular hydrogen bonding in the 2-isomer. This phenomenon effectively reduces the polarity of the 2-hydroxy compound resulting in a greater log P and hence greater retention than the 4-hydroxy analogue. The intermediate retention of the 3-isomer is difficult to rationalize. Overall however, the retention order is similar to that reported by other workers [7, 8] for compounds with phenyl rings di-substituted with H-bonding donor/acceptor groups.

Examination of the spectra of these four compounds gave a most surprising result. The 4-hydroxy isomer showed the characteristic bathochromic shift observed previously, accomplished by the loss of fine structure. In contrast the 2- and 3-isomers showed spectra which were superficially a hybrid between the parent and the 4-hydroxy analogue. The bathochromic shifts for the bands at *ca* 270 nm are only 6 and 4 nm (for the 2- and 3-isomer, respectively), in contrast to 18 nm for the 4-isomer. The 2- and 3-isomers also retained some of the fine structure observed in the spectrum of the parent compound (Fig. 6).

Although no aliphatic hydroxylated analogues were studied, the retention of these compounds would be much shorter than that of the isomeric 4-hydroxy compounds. This would result in a lower τ value for alkyl hydroxylation than the value of -0.59 observed here. This prediction is based on the good correlation between τ values and Hansch π values [7], and the fact that π values or there equivalent, for ring and aliphatic hydroxylation are -0.67 and -1.64, respectively [9], i.e. aliphatic hydroxy derivatives are much more polar than the isomeric ring hydroxy derivatives.

When used in combination it would appear that UV spectra and τ data could be particularly useful as an aid metabolite identification, enabling the hydroxylation at various sites to be distinguished.

Analysis of model compounds

It is a relatively simple matter to discriminate between various isomeric hydroxy derivatives on the basis of their UV spectra when all the compounds are available. When attempting to identify a metabolite of a novel compound such discrimination may not be as easy.

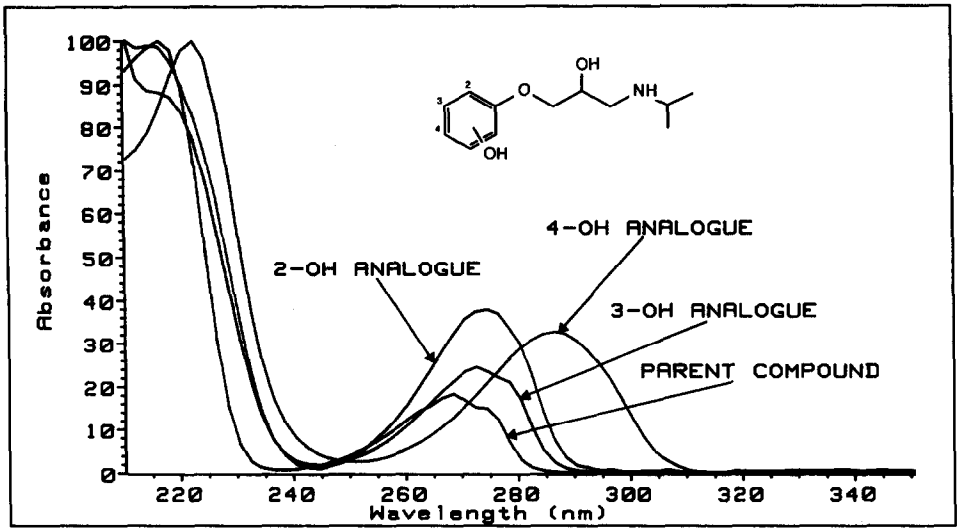

Figure 6
UV spectra and structures of 1-(isopropylamino)-3-(phenyloxy)-2-propanol and its three isomeric ring hydroxy analogues.

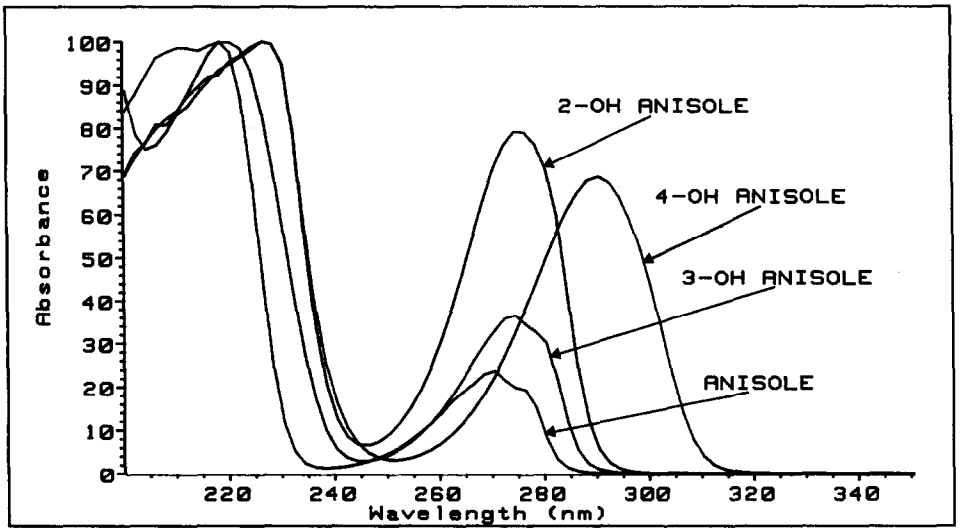

Figure 7
UV spectra of anisole and its three isomeric hydroxy derivatives.

However, some guidance may be obtained from the examination of analogues within the same series. When these are not available recourse can be made to readily available, simple model compounds which mimic the metabolic transformations under study. In this present work, anisole and its three isomeric hydroxy derivatives, were selected as being representative of the phenoxypropanolamines and their hydroxy metabolites.

The UV spectra for these compounds are shown in Fig. 7. Despite their simplicity in structure, the change in the UV spectra resulting from hydroxylation, and also the spectral change with the position of hydroxylation exactly mirror those seen with the more complex phenoxypropanolamines (Figs 4 and 6). Both the long and short wavelength peaks show the characteristic bathochromic shift, which is more pronounced in the 4-hydroxy analogue. Again the 2- or 3-substituted compounds show spectra which are essentially hybrids between the parent and the 4-hydroxy isomer. The loss in fine structure in the spectra of the 4-hydroxy compound is also evident.

Despite the simplicity of the anisole compounds, it is evident that they can be used as models to study the UV spectral changes in

more complex multifunctional phenoxypropanolamines.

Conclusions

This work clearly demonstrates how group contribution values (τ) taken in conjunction with UV spectral data can be used as an aid to the identification of hydroxy metabolites of phenoxypropanolamines. The procedure uses data collected under normal analytical procedures and unlike MS techniques does not require the use of expensive equipment and dedicated operators. This simple though powerful approach to metabolite identification has broad applicability and would appear to be worthy of further investigation and use.

A wide range of common metabolic transformations such as *O*-demethylation, *N*-dealkylation, *N*-oxide and *S*-oxide formation are amenable to analysis using the group contribution method [3]. Furthermore, when part of a UV chromophore, a number of these metabolic transformations could also result in characteristic UV spectral changes.

As a separate issue, this work also suggests that the analysis of UV spectral data is much under-rated. Until the advent of LC–MS and diode array detection most spectroscopic work was carried out off-line, usually following extensive sample clean up. Because of the undoubted power of mass and nuclear magnetic resonance spectroscopy, UV spectrometry has become a neglected tool in metabolite identification. However, the use of the DAD and other, conventional optics scanning detectors allows drug and metabolite spectra to be easily captured on-line. These data can then provide valuable information on the metabolite substitution pattern, as shown here, and occasionally can aid identification where MS has failed [P.J. Simons, personal communication]. UV spectra would appear to provide an untapped source of structural information for the metabolism chemist and more effort and training should be devoted to the interpretation of UV spectra.

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