Journal of Chromatography, 434 (1988) 363–375
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4480

DIRECT ABSORPTIOMETRIC QUANTITATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ABSENCE OF CONCOMITANT REFERENCE STANDARDS*

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SUMMARY

A method for absorptiometric quantification in high-performance liquid chromatography that utilizes the known molar absorption coefficients of the individual components of a mixture of analytes to enable a series of determinations to be carried out without the concomitant use of reference standards is described. An equation is derived that describes the dependence of the molar amount of a defined analyte on the parameters influencing the detector response and on the peak area of the analyte in the chromatogram. The equation can be used for the direct calculation of the molar amount of individual analytes in a well resolved mixture. A method for the analysis of two analytes, the peaks of which totally overlap but which differ by their molar absorption coefficients, is also described. The validity of the equations and the applicability of the proposed method was examined in the analysis of 5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene hydrochloride (benflurone) and its metabolites. Some examples of the application of this approach are considered.

INTRODUCTION

The column chromatographic determination of analytes that leave the column in separate concentration bands assumes proportionality between the total amount (m, mol) of analyte passing through the detector and the area of the analyte peak (P) on the chromatogram:

$$m = kP \quad (1)$$

As the slope k is generally not known in practice, and indeed can vary with the experimental conditions, a standard solution of the test substance at a defined

*Presented in part at the 6th Danube Symposium on Chromatography, Varna, October 12–17, 1987.

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concentration is subjected to concomitant analysis in each series of experiments (or, in the case of an internal standard, in each run).

An equation analogous to eqn. 1 holds between the amount of the standard (m_s) which has passed through the detector and its peak area (P_s):

$$m_s = k_s P_s \tag{2}$$

For identical solutes the value of k equals k_s, provided that both analyses have been carried out under identical conditions.

Dividing eqn. 1 by eqn. 2 allows k to be eliminated and a relationship is obtained which is routinely used, namely

$$m/m_s = P/P_s \text{ or } m = m_s(P/P_s) \tag{3}$$

Direct application of eqn. 1 for the quantification of substances without concomitant analysis of standards would be possible if the value of k were known. However, so far as the authors are aware, this approach has generally been neglected. The value of this coefficient depends on the conditions of chromatographic analysis, on the parameters that modify the detector response and on the nature of the analyte itself. In this study we have attempted to identify the parameters which contribute to the proportionality coefficient k in eqn. 1 in absorptiometric quantification. We have also examined the analytical applicability of eqn. 1 for direct quantification without concomitant running of standards, in cases when k is known or can be calculated.

THEORY

Single-component peaks

A standard column liquid chromatographic system with a UV detector, recorder and integrator was used. The flow of the concentration zones of each separated analyte through the detector is manifested as a change in absorbance as a function of time (or volume of mobile phase).

According to the Lambert–Beer law, given certain conditions, and at a defined

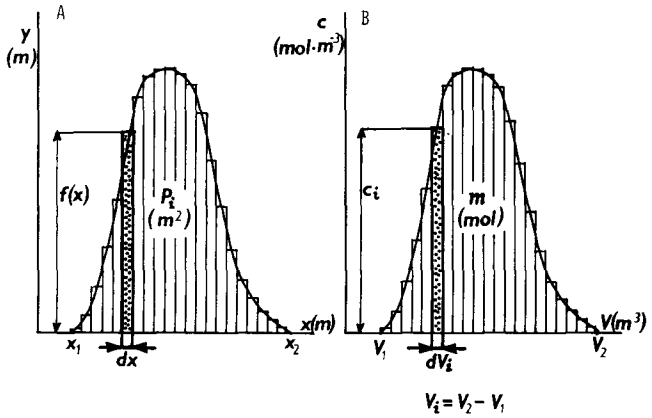


Fig. 1. Schematized chromatographic peak and definition of quantities.

wavelength λ, absorbance is proportional to analyte concentration. It should also be noted that the product of the volume element of the mobile phase, dV_i, and the concentration of analyte in that element, c_i, gives the amount of the substance, m_i, in this element of the concentration band.

The schematized chromatographic peak in Fig. 1 represents the elution profile of the concentration band of the analyte after it has left the column and entered the detector.

The shape of the chromatographic peak of the compound under study can be described by the general function $y=f(x)$, which is defined in the interval $(x_1;x_2)$ (see Fig. 1A). The area of the peak can be expressed by

$$P=\int_{x_1}^{x_2} f(x)dx \tag{4}$$

The dimensions of the same peak can also be expressed in units of concentration and volume (Fig. 1B). It is necessary to define following quantities:

V_i (m^3)	total eluate volume, corresponding to the concentration band of substance i (analyte) in the interval x_1 to x_2 (or V_1 to V_2) (cf. Fig. 1A and B)
t_i (s)	time necessary for V_i to pass through the detector
F ($m^3\ s^{-1}$)	flow-rate of the mobile phase
v_p ($m\ s^{-1}$)	velocity of the recorder chart paper
P_i (m^2)	peak area of the analyte
A_{FS}	absorbance corresponding to the full-scale recorder deflection
R (m)	maximum deflection of the pen (generally equivalent to the maximum width of the recorder chart)
A_i	local absorbance of the analyte i in the volume element dV of the concentration band (cf. Fig. 1A and B) at detection wavelength λ
ϵ_i^λ ($m^2\ mol^{-1}$)*	molar absorption coefficient of analyte i at λ
c_i ($mol\ m^{-3}$)	local concentration of the analyte i in the volume element dV_i
dm_i	instantaneous amount of analyte i in volume element dV_i
$f(x)$ (m)	vertical displacement of the pen on the chart paper corresponding to any position on the chart
L (m)	thickness of the light-absorbing layer in the detector cell
m_i (mol)	total molar amount of the analyte in the solute concentration band.

Let us first consider the quantities pertaining to the abscissa of the chromatogram (Fig. 1A and B). The recorder chart paper moves at the rate v_p, the distance dx of the chart paper is covered within the time interval dt:

$$dt=dx/v_p \tag{5}$$

*Conventionally, ϵ_i^λ is expressed as $1\ mol^{-1}\ cm^{-1}=10^{-1}\ m^2\ mol^{-1}$.

For any defined volume element of the eluate in which the concentration band of the eluate is present (Fig. 1B),

$$dV_i = F dt \tag{6}$$

By combining eqns. 5 and 6 we obtain

$$dV_i = (F dx)/v_p \tag{7}$$

Let us now consider the quantities pertaining to the ordinate of the chromatogram. Present-day UV detectors generally operate in calibrated absorbance ranges that are characterized by a preselected value of the maximum absorbance range A_{FS} (e.g. from 1.28 to 0.01 absorbance unit). Thus the output voltage of the detector which yields the maximum recorder deflection, R, corresponds to the declared value of A_{FS}. Generally, the deflection at any given point in the chromatographic profile corresponds to absorbance A_i. According to the Lambert–Beer law for specified conditions, this is proportional to the local concentration c_i of the analyte in the respective element of the concentration zone (Fig. 1B). Assuming the validity of the Lambert–Beer law at λ within the concentration range of the experiment, then at the centre of the time interval dt

$$A_{FS}/R = A_i/f(x) \tag{8}$$

As

$$A_i = \epsilon_i^{\lambda} c_i L \tag{9}$$

eqns. 8 and 9 can be rearranged to give

$$c_i = [A_{FS} f(x)]/(R\epsilon_i^{\lambda} L) \tag{10}$$

For the instantaneous molar amount of the analyte, dm_i, in the volume element dV_i (cf. Fig. 1B),

$$dm_i = c_i dV_i \tag{11}$$

which from eqns. 7 and 10 can be rearranged to give

$$dm_i = [(A_{FS}F)/(R\epsilon_i^{\lambda} L v_p)] f(x) dx \tag{12}$$

On integration

$$\int_0^m dm_i = [(A_{FS}F)/(R\epsilon_i^{\lambda} L v_p)] \int_{x_1}^{x_2} f(x) dx \tag{13}$$

This can be rearranged, using eqn. 4, to give

$$m_i = [(A_{FS}F)/(R\epsilon_i^{\lambda} L v_p)] P \tag{14}$$

Comparing eqns. 1 and 14 we see that the slope of the linear function k at λ is equivalent to

$$k = (A_{FS}F)/(R L v_p \epsilon_i^{\lambda}) \tag{15}$$

which can clearly be calculated with relative ease. In the Experimental section,

the applicability of eqn. 14 to the direct calculation of the molar amount of analyte in the concentration band leaving the column will be examined.

Overlapping peaks

One of the problems that can complicate the quantification of the mixture under analysis is insufficient resolution of two analytes. It can happen that two substances cannot be resolved in any of the systems that have been tried. This problem can be solved if radiation at two different wavelengths is used for the detection and quantification of combined concentration bands of two unresolved analytes. The underlying assumptions are of course, that the ratios of the molar absorption coefficients of the two compounds at the two wavelengths selected are sufficiently different, that no other absorbing compound occurs in the same time interval of the chromatogram, that neither analyte affects the light-absorption properties of the other and that each analyte obeys the Lambert–Beer law at the wavelengths concerned. The following quantities will be used in the derivations below:

$\lambda 1, \lambda 2$ two different wavelengths used for detection
$p^{\lambda 1}$ total area of the combined peaks of the unresolved analytes A and B at wavelength $\lambda 1$
$p^{\lambda 2}$ total area of the combined peaks of the unresolved analytes A and B at wavelength $\lambda 2$
$p_A^{\lambda 1}$ individual peak area of component A at $\lambda 1$
$p_B^{\lambda 1}$ individual peak area of component B at $\lambda 1$
$p_A^{\lambda 2}$ individual peak area of component A at $\lambda 2$
$p_B^{\lambda 2}$ individual peak area of component B at $\lambda 2$
$\epsilon_A^{\lambda 1}$ molar absorption coefficient of compound A at $\lambda 1$
$\epsilon_B^{\lambda 1}$ molar absorption coefficient of compound B at $\lambda 1$
$\epsilon_A^{\lambda 2}$ molar absorption coefficient of compound A at $\lambda 2$
$\epsilon_B^{\lambda 2}$ molar absorption coefficient of compound B at $\lambda 2$

If two absorbing components A and B coelute in the mobile phase flowing through the detector, the additivity law of the individual absorbance at λ of each of the components is valid at any point in the combined peak profile of compounds A and B. The total absorbance at any wavelength λ, measured at any point, is therefore

$$A = (\epsilon_A^{\lambda} c_A + \epsilon_B^{\lambda} c_B) L \tag{16}$$

where c_A and c_B are the instantaneous solute concentrations.

It can be shown relatively simply that the whole area of the combined peak of the unresolved compounds A and B will also equal the sum of the respective areas of the peaks of compound A and B:

$$P^{\lambda 1} = P_A^{\lambda 1} + P_B^{\lambda 1} \tag{17}$$

$$P^{\lambda 2} = P_A^{\lambda 2} + P_B^{\lambda 2} \tag{18}$$

It follows from eqn. 14 that for the same substance the ratios of the peak areas and molar absorption coefficients at one wavelength must equal the respective ratios at another wavelength:

$$P_A^{\lambda 1}/\epsilon_A^{\lambda 1} = P_A^{\lambda 2}/\epsilon_A^{\lambda 2} \quad (19)$$

$$P_B^{\lambda 1}/\epsilon_B^{\lambda 1} = P_B^{\lambda 2}/\epsilon_B^{\lambda 2} \quad (20)$$

Thus a set of four equations (eqns. 17–20) with four unknowns ($P_A^{\lambda 1}$, $P_B^{\lambda 1}$, $P_A^{\lambda 2}$ and $P_B^{\lambda 2}$) is produced. By repeated substitution they are solved to give

$$P_A^{\lambda 1} = (P^{\lambda 2} - P^{\lambda 1}\epsilon_B^{\lambda 2}/\epsilon_B^{\lambda 1})/(\epsilon_A^{\lambda 2}/\epsilon_A^{\lambda 1} - \epsilon_B^{\lambda 2}/\epsilon_B^{\lambda 1}) \quad (21)$$

$$P_B^{\lambda 1} = (P^{\lambda 2} - P^{\lambda 1}\epsilon_A^{\lambda 2}/\epsilon_A^{\lambda 1})/(\epsilon_B^{\lambda 2}/\epsilon_B^{\lambda 1} - \epsilon_A^{\lambda 2}/\epsilon_A^{\lambda 1}) \quad (22)$$

$$P_A^{\lambda 2} = (P^{\lambda 1} - P^{\lambda 2}\epsilon_B^{\lambda 1}/\epsilon_B^{\lambda 2})/(\epsilon_A^{\lambda 1}/\epsilon_A^{\lambda 2} - \epsilon_B^{\lambda 1}/\epsilon_B^{\lambda 2}) \quad (23)$$

$$P_B^{\lambda 2} = (P^{\lambda 1} - P^{\lambda 2}\epsilon_A^{\lambda 1}/\epsilon_A^{\lambda 2})/(\epsilon_B^{\lambda 1}/\epsilon_B^{\lambda 2} - \epsilon_A^{\lambda 1}/\epsilon_A^{\lambda 2}) \quad (24)$$

By using eqns. 21–24, the relative proportions of A and B can be ascertained. The calculated P_A^{λ} and P_B^{λ} values are then entered in eqn. 14 to obtain m_A and m_B.

EXPERIMENTAL

Benzofluorene derivatives (see Fig. 2)

The compounds used were 5-(2-dimethylaminoethoxy)-7-hydroxy-7*H*-benzo[*c*]fluorene N-oxide (reduced N-oxide, **1**), 5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene N-oxide (N-oxide, **2**), 5-(2-methylaminoethoxy)-7-hydroxy-7*H*-benzo[*c*]fluorene hydrochloride (reduced N-demethylated benflurone, **3**), 9-hydroxy-5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene hydrochloride (phenolic 9-hydroxybenflurone, **4**), 5-(2-dimethylaminoethoxy)-7-hydroxy-7*H*-benzo[*c*]fluorene hydrochloride (reduced benflurone, **5**), 5-(2-methylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene hydrochloride (N-demethylated benflurone, **6**) and 5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene hydrochloride (benflurone, **7**). Compounds **2** and **7** were prepared in the Research Institute for Pharmacy and Biochemistry in Prague (Czechoslovakia) and the others were prepared or isolated in our laboratory [1,2].

Mobile phase components

Acetonitrile (puriss. p.a. grade), octylamine (puriss. grade) (Fluka, Buchs, Switzerland), methanol, 2-propanol and phosphoric acid (p.a. grade, Lachema, Brno, Czechoslovakia) were used.

Chromatography (see Fig. 4)

The chromatographic set-up consisted of an HPP 4001 high-pressure pump (Laboratory Instruments, Prague, Czechoslovakia), a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), CGC columns (150 mm $\times$3.3 mm I.D.) containing Separon SGX C_{18} (5 μm) (Laboratory Instruments), an LC-3 UV detector (Pye Unicam, Cambridge, U.K.), a CI-100 computing integrator and a TZ 4221 line recorder (Laboratory Instruments).

The mobile phase was octylamine buffer–acetonitrile–2-propanol (2:2:1, v/v). The buffer was prepared by mixing 2 ml of *n*-octylamine with 990 ml of water, adjusting the pH to 7.4 with a solution of phosphoric acid (2 mol l^{-1}) and diluting to 1000 ml with water).

Spectrophotometer

SP 8-200 UV–VIS spectrophotometer (Pye Unicam) was used for the study of the spectra and the calculation of the molar absorption coefficients.

Computer

An IQ 151 computer was used for the calculations.

RESULTS

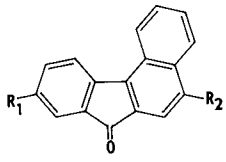
Analysis of benzofluorene compounds

Benflurone (Fig. 2, compound **7**), a new antineoplastic developed in the Research Institute for Pharmacy and Biochemistry in Prague by Křepelka and coworkers [3,4], is transformed by the microsomal fraction of liver homogenates of various animals into a number of metabolites [5–7]. The structures of the main metabolites are presented in Fig. 2 and were confirmed by the synthesis of reference compounds and comparison of their chromatographic and spectral characteristics (IR, mass and NMR spectroscopy) [1,2,7]. Optimum conditions (see Experimental) for the chromatographic separation and detection of these benzo[*c*]fluorene derivatives were selected on the basis of preliminary analyses of a model mixture of the metabolites (prepared by chemical synthesis).

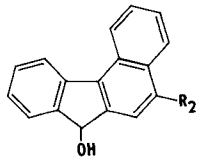
The separation of 7-dihydrobenflurone (**5**) and phenolic 9-hydroxybenflurone (**4**) remained a problem, as they exhibited closely similar retentions in all the systems tested so far. Quantification of these two derivatives was attempted by the approach involving eqns. 21–24 and 14.

The 7-oxo-7*H*-benzo[*c*]fluorene group includes substances **7**, **6** and **2**. Members of this group exhibit an exploitable UV absorption maximum at 294 nm (Fig. 3).

7-Hydroxy-7*H*-benzo[*c*]fluorenes include substances **5**, **3** and **1**. The UV absorption maximum of these three derivatives lies at 340 nm.



R_1	R_2	No.
H–	-O-$CH_2CH_2N(CH_3)_2$	7
HO–	-O-$CH_2CH_2N(CH_3)_2$	4
H–	-O-$CH_2CH_2NHCH_3$	6
H–	-O-$CH_2CH_2N(CH_3)_2O$	2



R_2	
-O-$CH_2CH_2N(CH_3)_2$	5
-O-$CH_2CH_2NHCH_3$	3
-O-$CH_2CH_2N(CH_3)_2O$	1

Fig. 2. Structures of benzo[*c*]fluorene analytes.

The third type is represented by the phenolic 9-hydroxybenflurone (**4**). The spectrum of this compound is analogous to those of the 7-oxo-7*H*-benzo[*c*]fluorenes of the first group.

On the basis of these spectral characteristics, the wavelengths chosen for monitoring the effluent were the absorption maxima of the first and second groups, 294 and 340 nm, respectively.

All analyses were carried out at both wavelengths. The agreement of both results was considered to be evidence of the correctness and applicability of the equations derived.

Single-component peaks

In order to check the validity and applicability of eqn. 14, one representative of each of the spectral types was selected. The standards of benflurone (**7**), 7-dihydrobenflurone (**5**) and phenolic 9-hydroxybenflurone (**4**) were purified by preparative thin-layer chromatography, reconverted to hydrochlorides, which crystallize well, and dried to constant weight over phosphorus pentoxide at 100°C. From these substances a stock solution ($2.5 \cdot 10^{-3}$ mol l^{-1}) in the mobile phase was prepared, from which the necessary concentrations were obtained by diluting with the mobile phase. UV spectra of these three benzo[*c*]fluorenes were recorded and their molar absorption coefficients measured (see Fig. 3 and Table I).

The maximum deflection of the recorder pen, R, should theoretically be 0.25 m (the detector output and recorder input were 10 mV; the height of the deflection on the recorder at an input of 10 mV should correspond to the maximum width of the registration paper, 0.25 m). This was verified by injecting a 100-fold diluted

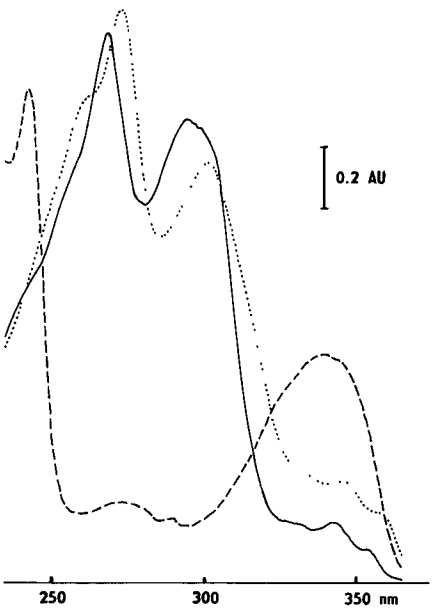


Fig. 3. UV spectra of benzo[*c*]fluorenes ($c = 5 \cdot 10^{-5}$ mol l^{-1}). Solid line, 7-oxo-7*H*-benzo[*c*]fluorenes; broken line, 7-hydroxy-7*H*-benzo[*c*]fluorenes; dotted line, 5-(2-dimethylaminoethoxy)-9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorene.

TABLE I

VALUES OF MOLAR ABSORPTION COEFFICIENTS OF BENZO[*c*]FLUORENES

Compound	ϵ_i' ($m^2\ mol^{-1}$)*	
	294 nm	340 nm
7-Oxo-7*H*-benzo[*c*]fluorenes	$2.94 \cdot 10^3$	$0.38 \cdot 10^3$
7-Hydroxy-7*H*-benzo[*c*]fluorenes	$0.37 \cdot 10^3$	$1.47 \cdot 10^3$
9-Hydroxy-7-oxo-7*H*-benzo[*c*]fluorenes	$2.44 \cdot 10^3$	$0.64 \cdot 10^3$

*See footnote in Theory section.

TABLE II

RESULTS EXEMPLIFIED FOR $m_t = 2.5 \cdot 10^{-8}$ mol INTRODUCED INTO THE CHROMATOGRAPH

m_c (mol) = analytical results calculated using eqn. 14; m_t (mol) = amount taken introduced into the chromatograph. Values constant for all experiments: $F = 8.33 \cdot 10^{-9}\ m^3\ s^{-1}$ ($= 0.5\ ml\ min^{-1}$), $L = 10^{-2}$ m and $v_p = 5 \cdot 10^{-5}\ m\ s^{-1}$.

Compound	λ (nm)	A_{FS}	P (m^2)	R (m)	ϵ' ($m^2\ mol^{-1}$)	m_c (mol)
Benflurone	294	1.28	$8.48 \cdot 10^{-4}$	0.242	$2.94 \cdot 10^3$	$2.54 \cdot 10^{-8}$
	340	1.28	$1.18 \cdot 10^{-4}$	0.263	$0.38 \cdot 10^3$	$2.58 \cdot 10^{-8}$
7-Dihydrobenflurone	294	1.28	$1.16 \cdot 10^{-4}$	0.262	$0.37 \cdot 10^3$	$2.55 \cdot 10^{-8}$
	340	1.28	$4.41 \cdot 10^{-4}$	0.247	$1.47 \cdot 10^3$	$2.59 \cdot 10^{-8}$
9-Hydroxybenflurone	294	1.28	$6.8 \cdot 10^{-4}$	0.246	$2.44 \cdot 10^3$	$2.42 \cdot 10^{-8}$
	340	1.28	$1.83 \cdot 10^{-4}$	0.251	$0.64 \cdot 10^3$	$2.43 \cdot 10^{-8}$

stock solution directly into the flow-through detector cell at both wavelengths (294 and 340 nm). The optical path in the flow cell was assumed to be 0.01 m, as described by manufacturer. From the pen deflection for this concentration relative to the baseline, corrected values of R were calculated and are given in Table II.

Once the values of the constants A_{FS}, F, R and v_p had been established, defined amounts of standards with known ϵ_i^λ were chromatographed (see Fig. 4). Peak-area counts obtained from the integrator were transformed into surface area, P (m^2), by means of a factor that had been calculated by comparison of the integrator printouts with the results obtained by approximating the areas recorded by the linear recorder by a series of triangles. This factor was calculated from three values each day. The variation of this factor within one day was negligible.

Examples of the calculations are presented in Table II for an amount of $m_t = 2.5 \cdot 10^{-8}$ of the three substances introduced on to the column in separate experiments. Amounts found by experiments and calculation (m_c) were obtained with the known (stoichiometric) amounts of substances (m_t) introduced onto the column.

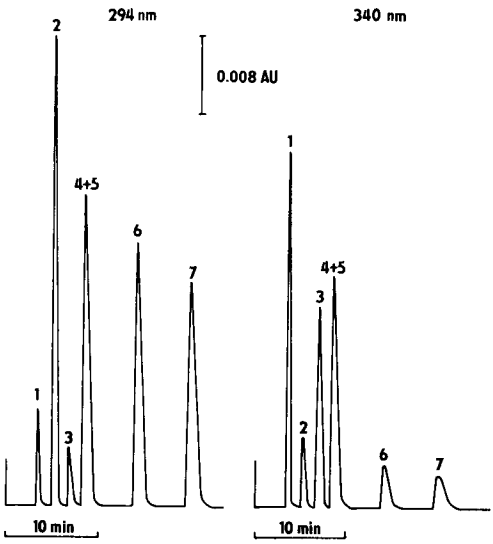


Fig. 4. Chromatograms of benzo[*c*]fluorenes ($4.17 \cdot 10^{-10}$ mol of each analyte) at 294 and 340 nm.

TABLE III

RECOVERIES FOR DIFFERENT AMOUNTS, m_t, INTRODUCED INTO THE CHROMATOGRAPH

m_t (mol)	Recovery ($a = m_c/m_t$)					
	Benflurone		7-Dihydrobenflurone		9-Hydroxybenflurone	
	294 nm	340 nm	294 nm	340 nm	294 nm	340 nm
$2.50 \cdot 10^{-8}$	1.016	1.031	1.020	1.036	0.968	0.972
$1.25 \cdot 10^{-8}$	0.973	1.110	0.952	0.984	1.088	0.944
$5.00 \cdot 10^{-9}$	1.032	1.046	0.978	0.986	1.004	1.048
$2.50 \cdot 10^{-9}$	1.068	1.088	0.972	0.988	0.992	1.068
$1.67 \cdot 10^{-9}$	1.038	0.948	0.972	1.026	1.116	0.984
$1.25 \cdot 10^{-9}$	1.088	1.120	0.889	0.968	1.104	1.008
$1.00 \cdot 10^{-10}$	–	–	1.130	1.181	–	–
$8.33 \cdot 10^{-10}$	1.031	1.040	0.991	0.996	1.028	1.046
$7.14 \cdot 10^{-10}$	1.023	1.046	–	–	1.029	1.046
$6.25 \cdot 10^{-10}$	1.022	1.030	0.982	0.989	1.072	1.090
$5.00 \cdot 10^{-10}$	1.026	1.048	–	–	0.972	1.012
$4.17 \cdot 10^{-10}$	1.024	1.031	1.007	1.012	1.074	1.084
$3.13 \cdot 10^{-10}$	1.021	1.040	0.993	0.986	1.120	1.135
$6.25 \cdot 10^{-11}$	0.989	1.003	0.982	0.986	1.066	1.125
Average	1.027	1.045	0.989	1.012	1.049	1.043
S.D.	0.029	0.044	0.055	0.057	0.053	0.058

Recoveries were expressed as $a = m_c/m_t$ and the averages and standard deviations were calculated. In Table III, recoveries are presented for all the amounts tested and both wavelengths.

TABLE IV

DETERMINATION OF 9-HYDROXYBENFLURONE (**4**) AND 7-DIHYDROBENFLURONE (**5**) IN MODEL BINARY MIXTURES WITH VARIOUS PROPORTIONS OF THE TWO COMPONENTS

$m_t(\mathbf{4})$ and $m_t(\mathbf{5})$ (mol) = molar quantities of components **4** and **5** in 10 μl of the mixture introduced on to the column; $P^{\lambda}(\mathbf{4}+\mathbf{5})$ (m^2) = experimentally derived total area of unresolved peaks of substances **4** and **5**; $P^{\lambda}(\mathbf{4})$ and $P^{\lambda}(\mathbf{5})$ (m^2) = areas calculated using eqns. 21–24 corresponding to substances **4** and **5**, respectively; $m_c(\mathbf{4})$ and $m_c(\mathbf{5})$ (mol) = molar amounts of substances **4** and **5**, respectively, calculated using eqn. 14.

$m_t(\mathbf{4})$ (10^{-9} mol)	$m_t(\mathbf{5})$ (10^{-9} mol)	λ (nm)	$P^{\lambda}(\mathbf{4}+\mathbf{5})$ (10^{-5} m^2)	$P^{\lambda}(\mathbf{4})$ (10^{-5} m^2)	$P^{\lambda}(\mathbf{5})$ (10^{-5} m^2)	$m_c(\mathbf{4})$ (10^{-9} mol)	$m_c(\mathbf{5})$ (10^{-9} mol)
15.83	0.83	294	44.57	44.2	0.37	15.72	0.815
		340	13.2	11.8	1.41	15.67	0.826
15.00	1.66	294	41.8	41.1	0.7	14.6	1.54
		340	13.21	10.5	2.71	13.9	1.59
12.50	4.17	294	35.0	33.2	1.84	11.8	4.05
		340	15.6	8.58	7.01	11.4	4.12
8.33	8.33	294	27.0	23.3	3.74	8.29	8.22
		340	19.93	6.13	13.8	8.14	8.12
4.17	12.50	294	17.7	11.6	6.1	4.11	13.4
		340	25.03	3.03	22.0	4.02	12.9
1.67	15.00	294	10.6	4.28	6.32	1.52	13.9
		340	25.4	1.11	24.3	1.48	14.3
0.83	15.83	294	8.97	2.37	6.6	0.842	14.5
		340	26.33	0.63	25.7	0.839	15.1

Overlapping peaks

Solutions of mixtures of 7-dihydrobenflurone (**5**) and phenolic 9-hydroxybenflurone (**4**) in various proportions were prepared and chromatographed. From the combined peak of the two substances the molar amounts of each component in the resolved mixture were calculated using eqns. 21–24 and 14 (Table IV). Again, the results were compared with m_t, the stoichiometric amounts of both substances introduced on to the column (Table V), and similar calculations of recoveries were carried out.

DISCUSSION

The systematic and random errors (see Table III) were acceptable for the biotransformation studies for which the method was intended. There seemed to be a bias towards more than theoretical recoveries ($a > 1$), the reasons for which we did not study.

Table V shows that the error did not increase when unresolved binary peak areas were split into contributions from individual analytes on the basis of results for two different wavelengths, and the masses calculated from these contributions.

In comparison with the usual strategy of using external or internal standards, the proposed method is not more complicated once the constants describing the set-up to be used (A_{FS}, v_p, R and L) and the relationships between integrator

TABLE V

RECOVERIES FOR 9-HYDROXYBENFLURONE (**4**) AND 7-DIHYDROBENFLURONE (**5**) CALCULATED FROM VALUES OF m_c AND m_t IN TABLE IV

m_t(**4**) (10^{-9} mol)	m_c(**5**) (10^{-9} mol)	Recovery ($a = m_c/m_t$)			
		9-Hydroxybenflurone (**4**)		7-Dihydrobenflurone (**5**)	
		294 nm	340 nm	294 nm	340 nm
15.83	0.83	0.993	0.9897	0.978	0.992
15.00	1.66	0.973	0.927	0.928	0.958
12.50	4.17	0.944	0.912	0.972	0.989
8.33	8.33	0.995	0.977	0.987	0.975
4.17	12.50	0.987	0.965	1.072	1.032
1.67	15.00	0.916	0.892	0.927	0.953
0.83	15.83	1.011	1.007	0.916	0.954
Average		0.974	0.953	0.969	0.979
S.D.		0.033	0.043	0.054	0.028

output and surface area on the recorder chart have been established, molar absorption coefficients of the analytes obtained from spectrophotometer assays and F estimated for the particular run. The calculation is more involved with overlapping peaks of spectrally different substances than with peaks of single compounds, but the same would apply to such calculations if standards were used concomitantly.

We do not argue that the approach proposed is generally preferable. We think, however, that it is of advantage in some cases. For example, it makes it possible to check for substantial losses during chromatography caused by irreversible adsorption or degradation in the column bed. Once these losses have been demonstrated by significantly low recoveries, their causes could be sought and possibly prevented, thus improving the chromatographic method employed. Prolonged tailing, resulting from a strongly convex isotherm (due, possibly, to a limited number of strongly adsorbing sites) or from slow desorption, can also lead to apparent losses, as the integration would not include the whole area of the tail within the peak area.

The calculation of the amount of two analytes within overlapping peaks was facilitated by the dissimilarity of the spectra of the keto and hydroxy compounds (Fig. 3). The determination of the limits of applicability of eqns. 21–24 would need a special study to define the minimum difference of ϵ^{λ} values at two wavelengths.

Recoveries higher than 100%, if established by this approach, would also draw attention to irregularities of the chromatographic procedure. They may result from a displacement of light-absorbing material remaining in the column from previous analyses.

The proposed method has been used routinely for analytical monitoring of the metabolism of benflurone in vitro (using microsomes, 10 000 *g* supernatant or

isolated hepatocytes) or in vivo (urine, faeces), and the results obtained demonstrated the practical applicability of the method. In order to keep the length of this paper within reasonable limits, we intend to publish these data elsewhere.

ACKNOWLEDGEMENT

The authors would like to thank Professor A.F. Fell (Bradford, U.K.) for comments.

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