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EVALUATION OF PHOTOMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DATA FOR THE DETERMINATION OF BENFLURONE METABOLITES IN BIOLOGICAL MATERIALS WITH AND WITHOUT CONCOMITANT USE OF A STANDARD

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SUMMARY

The results produced by a new method of calculation based on the knowledge of the absorption coefficient and instrumental parameters without the concomitant use of a standard were compared with those calculated by the routine external standard method for a model system utilizing the quantification of benzofluorene derivatives. These were present in the incubation mixture of 5-[2-(dimethylamino)ethoxy]-7-oxo-7*H*-benzo[*c*]fluorene (benflurone) with the microsomal fraction of rat liver homogenate. Reasonable agreement between the two methods was observed. The potential utility of the new method of calculation is discussed.

INTRODUCTION

In a preceding paper[1], an equation which describes the relationship between the molar amount of an individual analyte and the conditions and parameters of the liquid chromatographic analysis was derived. The validity and applicability of this equation were verified by analysing a model mixture of benzofluorenes, separated by liquid chromatography. If the molar absorption coefficient, ε_i^λ, of each individual analyte i were known at a specified wavelength λ, and if certain instrumental parameters were defined, it was found possible to calculate the molar amount of each individual component in the mixture without the requirement for chromatographing known amounts of a reference compound or compounds, either in the same run (*i.e.*, internal standard) or in parallel runs (*i.e.*, external standard).

The instrumental parameters to be defined are constant, or at least they can be easily and precisely selected or measured, *i.e.*, optical path length in the flow cell, full-scale absorbance range, maximum deflection of the recording integrator pen for the selected absorbance range, velocity of the recording integrator chart paper and chromatographic peak area.

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The use of multi-channel detection opens up a number of new possibilities for processing the chromatographic data, including a range of techniques for assessing the purity of an individual peak[2]. Some of these techniques could be used in conjunction with the proposed methods for quantification without the use of concomitant standards.

The use of concomitant reference standards in chromatographic analysis has been generally preferred for its well known advantages. However, the method of quantification without the use of a standard introduces some new possibilities, which complement methods based on standards in the usual way. Two examples of the potential utility of the proposed method are discussed here in general terms.

Check for recovery on the column: the irreversible or slowly reversible sorption or degradation of the analyte on the column cannot be ascertained with reference to the standards, as the latter would also undergo proportional losses. Low recovery can be demonstrated by comparing the amounts introduced into the column and the results of the analysis which do not rely on concomitantly run standards. Low recovery should be a stimulus for revising the procedure, as it might be accompanied by a lack of reproducibility.

Standards not available: the analyst may have to cope with the task of quantifying, with the help of high-performance liquid chromatography (HPLC), substances for which the reference samples are not available and for which related compounds, which could serve as non-identical standards, have not been chosen. This task becomes especially important when the identity of a peak is not known, but where a search is being conducted to establish it with the help of a database containing such parameters as extractibility at various pH values, chromatographic retention (HPLC, thin-layer chromatography) and UV–VIS and other spectra. Of course, in practice, the database would have to be limited to a certain class, such as drugs, their known metabolites, pesticides, etc. Comparison with the database may generate a probability that a certain peak belongs to one compound or one of a group of compounds. If the retention data for a certain number of selected chromatographic systems and absorption coefficients for the respective solvent mixtures at certain selected wavelengths were also included in the database, it should be possible to quantify the amount of the substance(s) considered to be responsible for the respective peak. It is more likely that these parameters would be available than would reference samples of all the substances themselves.

If, in a simpler case, the database consisted only of the retention parameters and UV–VIS spectra (obtainable using a photodiode-array detector) in certain chromatographic and solvent systems, the identification of the peaks and the quantification of the compounds suspected to be responsible for them could be achieved automatically using suitable software (overlapping peaks would certainly represent a complication in this approach).

An objection can, of course, be raised against this suggestion, namely that if incomplete recovery is considered possible, any quantification which would assume complete recovery without a check can at best be considered an estimate. Checks for recovery would thus have to be performed beforehand. The possibility of degradation during chromatography can be assessed by considering the chemistry of the compound. Irreversible (or slowly reversible) sorption should be similar within a group of physico-chemically related substances (with respect to their hydrophobi-

city, acid–base and polar properties and possible amphiphilic character). It would therefore not be necessary to check all substances for recovery in the chromatographic system employed, but only representative examples of such groups.

In this paper, the results of quantitative analyses of biological samples evaluated by the proposed method are compared with those obtained by the routine method using an appropriate external standard. Examples of analyses of the metabolites of benflurone (a potential cytostatic drug) with rabbit liver microsomal fraction[3] are presented.

THEORY

If, in a series of analyses, all parameters in the following previously derived equation (the individual symbols are defined at the end of the paper):

$$m_i = [(A_{FS}F)/(R\varepsilon_i^{\lambda}Lv_p)]P_i \tag{1}$$

are constant, except for P_i, it is possible to combine these factors to give a proportionality constant, K_1 (or K_2).

If it was necessary to quantify two compounds A and B which eluted closely together and differed sufficiently with respect to their molar absorption coefficients at two wavelengths, λ_1 and λ_2, the values of the calculated peak areas, $P_A^{\lambda 1}$, $P_A^{\lambda 2}$, $P_B^{\lambda 1}$ and $P_B^{\lambda 2}$, derived previously[1], were introduced into eqn. 1 instead of the value P_i, cf. eqns. 2–5:

$$P_A^{\lambda 1} = (P^{\lambda 2} - P^{\lambda 1}\varepsilon_B^{\lambda 2}/\varepsilon_B^{\lambda 1})/(\varepsilon_A^{\lambda 2}/\varepsilon_A^{\lambda 1} - \varepsilon_B^{\lambda 2}/\varepsilon_B^{\lambda 1}) \tag{2}$$

$$P_B^{\lambda 1} = (P^{\lambda 2} - P^{\lambda 1}\varepsilon_A^{\lambda 2}/\varepsilon_A^{\lambda 1})/(\varepsilon_B^{\lambda 2}/\varepsilon_B^{\lambda 1} - \varepsilon_A^{\lambda 2}/\varepsilon_A^{\lambda 1}) \tag{3}$$

$$P_A^{\lambda 2} = (P^{\lambda 1} - P^{\lambda 2}\varepsilon_B^{\lambda 1}/\varepsilon_B^{\lambda 2})/(\varepsilon_A^{\lambda 1}/\varepsilon_A^{\lambda 2} - \varepsilon_B^{\lambda 1}/\varepsilon_B^{\lambda 2}) \tag{4}$$

$$P_B^{\lambda 2} = (P^{\lambda 1} - P^{\lambda 2}\varepsilon_A^{\lambda 1}/\varepsilon_A^{\lambda 2})/(\varepsilon_B^{\lambda 1}/\varepsilon_B^{\lambda 2} - \varepsilon_A^{\lambda 1}/\varepsilon_A^{\lambda 2}) \tag{5}$$

EXPERIMENTAL

Materials

Benzo[c]fluorene derivatives. The formulae of the benzo[*c*]fluorenes considered were given in the preceding paper[1]. Their names are given at the end of the paper (symbols), numbered in order of their appearance in the chromatogram (Fig. 1). Reference standards of substances **2** and **7** were obtained from the Research Institute of Pharmacy and Biochemistry (Prague, Czechoslovakia)[4–6]. The remaining standards were obtained in the authors' laboratory by synthesis or isolation from biological material[7–9]. Reference substances **3–7** were in the hydrochloride form.

Mobile phase components. Acetonitrile, octylamine (puriss. p.a.; Fluka, Buchs, Switzerland), methanol, 2-propanol (analytical-grade, redistilled) and phosphoric acid (analytical-reagent grade) (Lachema, Brno, Czechoslovakia) were used.

Chromatography

The chromatographic equipment consisted of an HPP 4001 high-pressure pump (Laboratory Instruments, Prague, Czechoslovakia), Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), CGC compact glass columns (150 × 3.3 mm I.D.) with octadecylsilylated silica gel SGX C_{18}, 5 μm (Laboratory Instruments), a PU 4021 multi-channel photodiode-array UV–VIS detector (Pye Unicam, Cambridge, U.K.) and a PU 4810 computing integrator (Pye Unicam).

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

Computation

Calculations were carried out using an IBM-compatible AT computer (Falcon Technol., Kent, WA, U.S.A.). To speed up the calculations and their visual presentation, software in GWBASIC was written and is available from the authors on request.

Before starting a series of experiments it is advisable to check the value of R (which, for the PU 4810 integrator, should theoretically be 0.2) and to use the corrected value in the calculation. A method to establish R was described previously[1]. This is carried out with $A_t = 1.024$ V, since the maximum output potential from the detector is 1 V. With a different A_t value, such as 0.512 V, it is advisable to check the accuracy of this parameter by feeding the required value (0.512 V) from an external, well calibrated, stabilized source of direct potential and measuring the deflection of the pen, R(m).

It is necessary to derive the relationship between the arbitrary number which expresses the peak area (AREA) in the report of the computing integrator and the actual peak area in metric units (m^2). Several approaches are available.

(a) The "area-weighing" method is simplest. By repeatedly injecting a given amount of a substance onto the column, a sequence of peaks are generated which are then carefully cut out and weighed on an analytical balance. The average area of the peak in integrator units per gram of graph paper can then be calculated. We then measure the mass of well defined areas (*e.g.*, squares) drawn on and cut from the same graph paper and calculate the average area per gram of paper ($m^2\ g^{-1}$). By comparing the value of the average peak area per mass unit (integrator units g^{-1}) and the $m^2\ g^{-1}$ values, we find the relationship between integrator units and metric units. In our case one integrator unit corresponded to $7.4188 \cdot 10^{-12}$ m^2. The area expressed by an AREA number in the integrator report had therefore to be multiplied by $k = 7.4188 \cdot 10^{-12}$ to yield the area in metric units.

(b) As the PU 4810 integrator is programmable in modified BASIC, it is possible to generate squares of different sizes for the above measurement using a simple program:

```
10 INPUT "X,Y,A";X,Y,A: C=4.75
20 CHANAT ON: GRAPH
30 GRAPH C*X,Y,1
40 GRAPH C*X,Y+A,0: GRAPH C*(X+A),Y+A,0
50 GRAPH C*(X+A),Y,0: GRAPH C*X,Y,0
60 CHANAT OFF
70 END
```

TABLE I

MOLAR ABSORPTION COEFFICIENTS OF BENZO[*c*]FLUORENES

Compound	ε_i^{μ} (m^2 mol^{-1})	
	294 nm	*340 nm*
7-Oxo-7*H*-benzo[*c*]fluorenes (**2, 6, 7**)	2940	380
Phenolic compound **4**	2440	640
7-Hydroxy-7*H*-benzo[*c*]fluorenes (**1, 3, 5**)	370	1470

After switching on RUN, it is necessary to enter the coordinates of the first corner of the square (X, Y) and the length of the side of the square (A) in dimensionless units which do not correspond to integration units (*e.g.*, X, Y, A 100, 100, 263). The integrator then draws the required square.

When the results were evaluated by the proposed method[1], eqn. 1 and, in the case of overlapping peaks of substances **4** and **5**, eqns. 2–5 were used. These equations were also tentatively applied for substances **2** (see Discussion).

Table I gives the molar absorption coefficients employed in the calculation. Table II demonstrates the calculation by means of eqn. 1 using benflurone (**7**) of known concentration as an example. A_t was set to a nominal value of 1024 mV. The last column indicates recoveries which deviate only slightly from unity, in agreement with the conclusions from Table III in ref. 1.

When the results had to be evaluated by the external standard method, benflurone (**7**) was used as a reference. As the molar absorption coefficients of all the metabolites and benflurone at 294 and 340 nm are known, it was not necessary to employ further standards. Eqn. 6 was applied

$$m_i = m_s(A_r/\varepsilon_i^{\lambda})/(A_{r,s}/\varepsilon_s^{\lambda}) \tag{6}$$

For a series of experiments a proportionality constant, K_3, could be used. In the case of overlapping peaks, a set of equations similar to eqns. 2–5 was employed.

TABLE II

CALCULATION ACCORDING TO EQN. 1 EXEMPLIFIED WITH BENFLURONE (7) SAMPLES OF KNOWN CONCENTRATION

Constant values: $F = 1$ ml $min^{-1} = 1.66 \cdot 10^{-8}$ m^3 s^{-1}; $L = 0.01$ m; $P_i = 7.4188 \cdot 10^{-12}$ A_r m^2; $R = 0.1811$ m; $U = 1$ V; $V_p = 0.5$ cm $min^{-1} = 8.333 \cdot 10^{-5}$ m s^{-1}; $\varepsilon^{294} = 2940$ m^2 mol^{-1}.

m^a *(mol)*	A_{FS}	A_r	m_c^a *(mol)*	m_c/m_t
$1.250 \cdot 10^{-8}$	1.28	$37.846 \cdot 10^6$	$1.35 \cdot 10^{-8}$	1.08
$6.250 \cdot 10^{-9}$	0.64	$35.436 \cdot 10^6$	$6.32 \cdot 10^{-9}$	1.01
$3.125 \cdot 10^{-9}$	0.32	$36.108 \cdot 10^6$	$3.22 \cdot 10^{-9}$	1.03
$1.563 \cdot 10^{-9}$	0.16	$34.538 \cdot 10^6$	$1.54 \cdot 10^{-9}$	0.99
$7.813 \cdot 10^{-10}$	0.08	$34.808 \cdot 10^6$	$7.76 \cdot 10^{-10}$	0.99
$3.906 \cdot 10^{-10}$	0.04	$36.781 \cdot 10^6$	$4.1 \cdot 10^{-10}$	1.05

[a] m_t = amount of **7** injected onto the column; m_c = amount of **7** calculated using eqn. 1.

Incubation, extraction and analysis

Biotransformation of benflurone *in vitro* using the microsomal fraction of the rabbit liver homogenate was carried out according to ref. 3. The mixture of defined amounts of benflurone (**7**), the microsomal fraction and coenzymes was incubated for 30 min at 37°C in a suitably buffered aerated medium. The ensuing mixture of benflurone and its metabolites was extracted at pH 9–10 into ethyl acetate and evaporated *in vacuo* at maximally 40°C. The residue was dissolved in 1 ml of the mobile phase and analysed. Volumes of 10 μl of this solution were injected onto the chromatographic column and analysed at 294 and 340 nm (Fig. 1). In order to check the identity of the individual analytes passing through the detector, their spectra were recorded at the chromatographic peak maxima.

RESULTS

For the proposed evaluation method (eqn. 1), parameters given at the top of Table II were applied with the exception of $F = 8.333 \cdot 10^{-9}$ m^3 s^{-1}. $A_{FS} = 0.64$ was used (except for the values marked with asterisks in Table IV, for which $A_{FS} = 1.28$ was used).

The appropriate proportionality constants K_2 were calculated (Table III). For the oxo compounds **2, 6** and **7** and $\lambda = 294$ nm, the calculation is given here as an example: $K_2^{294} = A_{FS}Fk/(R\varepsilon_i^{294}Lv_p) = 0.64 \cdot 8.333 \cdot 10^{-9} \cdot 7.4188 \cdot 10^{-12}/(0.1811 \cdot 2940 \cdot 0.01 \cdot 8.333 \cdot 10^{-5}) = 8.9176 \cdot 10^{-17}$.

In the external standard method, $3.125 \cdot 10^{-9}$ mol of benflurone (**7**) gave a reading of $A_r = 35.835 \cdot 10^6$ at $\lambda = 294$ nm. The proportionality constants K_3 were calculated (Table III). For the oxo compounds **2, 6** and **7** at $\lambda = 294$ nm the following calculation applied: $K_3^{294} = m_s\varepsilon_s^{294}/(\varepsilon_i^{294}A_{r,s}) = 3.125 \cdot 10^{-9}/35.835 \cdot 10^6 = 8.72041 \cdot 10^{-17}$.

Differences between the corresponding K_2 and K_3 values (Table III) are, in the autors' opinion, acceptable.

Both methods of evaluation were applied to the HPLC absorptiometric analysis of the biotransformation mixture (5 μmol of **7** were incubated). The analyses were performed at two wavelengths, 294 and 340 nm (Table IV). Substances **1** and **3** were not quantified, as the small areas of their peaks would introduce serious errors irrespective of the method of calculation.

As compounds **4** and **5** were not well resolved, their AREA reports were summed and evaluated by the method for overlapping peaks of substances differing in the

TABLE III

PROPORTIONALITY CONSTANTS K_2 (PROPOSED METHOD) AND K_3 (EXTERNAL STANDARD METHOD) (10^{-17} mol m^{-2})

Compound	K_2		K_3	
	294 nm	*340 nm*	*294 nm*	*340 nm*
2, 6, 7	8.918	68.990	8.720	68.747
4	10.745	40.965	10.507	40.059
5	70.859	17.835	69.292	17.441

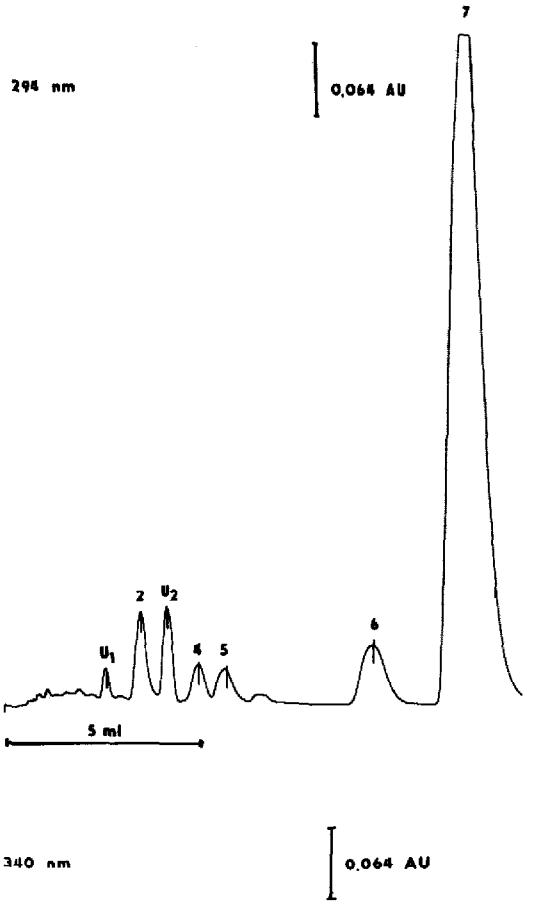

Fig. 1. Column liquid chromatogram of an aliquot of the extract from the incubate of 5 μmol of benflurone (7) with 9 μmol of NADPH. Detection at 294 and 340 nm, as indicated. Peak numbers correspond to compounds listed at the end of the paper under Symbols. U_1 and U_2 not characterized. See text for details on incubation and analysis.

TABLE IV

COMPARISON BETWEEN THE PROPOSED METHOD WITHOUT THE CONCOMITANT USE OF STANDARDS AND THE EXTERNAL STANDARD METHOD (INCUBATION WITH NADPH)

Results (m_i) are given in mol · 10^{-9} per sample: results a, calculated using the proposed method (eqn. 1), $F = 8.333 \cdot 10^9$ $m^3 s^{-1}$, $A_{FS} = 0.64$ (with the exception of the values marked with asterisks, which were measured at $A_{FS} = 1.28$), other parameters as in Tables I and II, A_t set nominally at 1024 mV; results b, calculated using the method as for results a including eqns. 2–5 for unresolved peaks; results c, calculated using the external standard method (eqn. 6), the amount of $3.125 \cdot 10^{-9}$ mol of benflurone corresponding to $A_t = 35\ 835\ 452$ (average of seven measurements under the same conditions as for the analyses of the samples); results d, same as c using a set of four equations analogous to eqns. 2–5 for unresolved peaks. A_r values are given in integrator units · 10^{-6}.

Sample with NADPH	*Result*	*λ (nm)*	*Benflurone N-oxide (2)*	*Hydroxy-benflurone (4)*	*Dihydro-benflurone (5)*	*Demethylated benflurone (6)*	*Benflurone (7)*
3	A_r	294	23.711	7.411	6.716	18.824	140.733*
		340	3.944	2.284	20.546	2.665	39.946
	a	294	2.11	0.79	4.76	1.68	25.1
		340	2.72	0.94	3.66	1.84	27.6
	b		2.09	0.96	3.65		
	c	294	2.09			1.64	24.5
		340	2.69			1.80	26.9
	d		2.05	0.94	3.57		
6	A_r	294	16.984	5.695	4.665	14.847	215.467
		340	4.121	3.771	16.089	2.155	31.756
	a	294	1.52	0.61	3.31	1.32	19.2
		340	2.84	1.54	2.87	1.49	21.9
	b		1.47	0.62	3.27		
	c	294	1.48			1.28	18.8
		340	2.78			1.45	21.4
	d		1.44	0.60	3.20		
9	A_r	294	11.222	5.926	6.363	15.083	126.716*
		340	1.321	2.444	18.277	2.176	33.995
	a	294	1.00	0.64	4.5	1.35	22.6
		340	0.91	1.00	3.26	1.50	23.5
	b		1.00	0.81	3.34		
	c	294	0.98			1.31	22.1
		340	0.89			1.46	22.9
	d		0.98	0.79	3.27		
12	A_r	294	11.740	5.581	5.860	12.702	119.987*
		340	2.167	2.793	17.055	1.876	32.379
	a	294	1.05	0.60	4.15	1.13	21.4
		340	1.50	1.14	3.04	1.29	22.3
	b		1.03	0.74	3.22		
	c	294	1.02			1.10	21.0
		340	1.46			1.26	21.8
	d		1.01	0.73	3.15		

shapes of their spectra, eqns. 2–5 (results b). An analogous approach was used in the internal standard method (results d).

For reasons to be discussed below, the results for compound **2** were also calculated under the assumption that its peak is contaminated with a substance of the

7-hydroxy-7*H*-benzo[*c*]fluorene type (Table I). Eqns. 2–5 and similar equations in the external standard method were used (results b and d).

DISCUSSION

As follows from the close similarity of the constants K_2 and K_3 (Table III), Table IV shows reasonable agreement within the pairs of values for identical samples calculated by the proposed method (eqn. 1), and with the external standard (eqn. 6).

The disagreement between the results using 294 and 340 nm for compound **2** with 6 and 12 μmol NADPH (Table IV, results a and c) was a matter of concern. The most likely explanation would be the possibility of impurities in the peaks, *i.e.*, the presence of other substances that have different absorption coefficient ratios for the two respective wavelengths than the substances to which the peak is attributed. Possible candidates are the matrix, containing extraneous substances from the incubation medium (microsomal constituents, coenzymes and their metabolites), and benflurone metabolites that have not been considered or even have not yet been characterized.

Chromatograms at 294 and 340 nm of a blank (the extract from an incubation of 6 μmol NADPH with the microsomal fraction without **7**) showed no peak in the position of compound **2**, so the matrix does not seem to be responsible. Assuming that the peak of the ketone **2** is contamined with a 7-hydroxy-7*H*-benzo[*c*]fluorene derivative (*cf.*, molar absorption coefficients in Table I, compound **5**), eqns. 2–5 and their counterparts in the external standard calculation method were employed. The results, given under b and d in Table IV, are closely similar to those under a and c for 294 nm, the absorption maximum of the ketone **2**.

Compounds **4** and **5** (phenolic hydroxybenflurone and dihydrobenflurone) were reported previously[1] to form a fused peak so that the use of eqns. 2–5 was necessary. In this work, these peaks were partially resolved owing to the slightly different chromatography properties of the columns, which had been in use for a long time. The amounts of these solutes were therefore calculated separately in Table IV under a. However, we consider the results obtained using eqns. 2–5, shown under b, to be more correct. For the evaluation with an external standard an analogous approach (d) was employed for these two solutes. The source of error for partially overlapping peaks (Table IV, a) seems to be the unreliable definition of the boundary between the peaks; in cases in which, at a given wavelength, the value for one of the compounds with method a is higher than that with method b (or d), the value for the other compound is lower.

Peaks U_1 and U_2, the chemical identities of which were not established, were not quantified. U_1 shows, at the apex of its peak, a spectrum reminiscent of oxo compounds (**2, 6, 7**), and therefore it cannot be attributed to compound **1**. The spectrum recorded near the apex of the chromatographic peak U_2 showed maxima at 268 and 308 nm (similar to those in the spectrum of compound **4**, but unlike the spectrum of compound **3**).

It can be concluded that the accuracy of the proposed method depends, as in the external standard method, on the resolution and purity of the peaks and on the proper selection of the sensitivity parameters (A_{FS} and A_t) for each peak.

The analyses were performed using equipment incorporating a PU 4021 multi-channel photodiode-array detector and a PU 4810 computing integrator. The

latter permits additional control of the signal from the detector, A_t (attenuation), which can be set to give a full-scale potential from 0.5 to 4096 mV. In the present experiments, however, A_t was kept constant at 1.024, in which case the maximum output U was 1 V, and eqn. 1 was used. If a prospective user of the method intended to vary A_t, the following equation would replace eqn. 1:

$$m_i = (A_t/U)[(A_{FS}F)/(R\varepsilon_i^{\lambda}Lv_p)]P_i \quad (7)$$

The xenobiochemical consequences of the dependence of the yield of the metabolites on the concentration of the coenzyme NADPH as a cosubstrate will be dealt with elsewhere[10].

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SYMBOLS

A_{FS}	absorbance corresponding to full-scale deflection of the recorder;
A_r	peak areas in arbitrary units (AREA report);
$A_{r,s}$	peak area of the external standard (arbitrary units); *cf.*, eqn. 6;
A_t (mV)	attenuation (maximum full-scale potential);
F (m^3 s^{-1})	flow-rate of the mobile phase;
$k = P_i/A_r$	proportionality constant between areas in metric and arbitrary units;
$K_1 = A_{FS}F/(R\varepsilon_i^{\lambda}Lv_p)$ (mol m^{-2})	proportionality constant for simplifying the calculation of $m_i = K_1P_i$ by the proposed method;
$K_2 = kK_1$ (mol m^{-2})	proportionality constant for simplifying the calculation of $m_i = K_2A_r$ by the proposed method;
$K_3 = m_s\varepsilon_s^{\lambda}/(\varepsilon_i^{\lambda}A_{r,s})$	proportionality constant for simplifying the calculation of $m_i = K_3A_r$ by the external standard method;
L (m)	thickness of the light-absorbing path in the detector cell;
m_i (mol)	molar amount of the analyte in a solute band;
m_s (mol)	molar amount of the external standard, eqn. 6;
P_i (m^2)	peak area of the analyte, eqns. 1 and 7;
$P^{\lambda 1}$, $P^{\lambda 2}$ (m^2)	total areas of the combined peaks of the unresolved analytes A and B at wavelengths λ_1 and λ_2, respectively, eqns 2–5;
R (m)	maximum deflection of the pen (generally equivalent to the maximum width of the recorder chart);
U (V)	maximum output potential from the detector;

v_p (m s^{-1})	velocity of the recorder chart paper;
ε_i^λ (m^2 mol^{-1})	molar absorption coefficient of analyte i at wavelength λ[a];
$\varepsilon_A^{\lambda 1}$, $\varepsilon_A^{\lambda 2}$, $\varepsilon_B^{\lambda 1}$, $\varepsilon_B^{\lambda 2}$ (m^2 mol^{-1})	molar absorption coefficients of compounds A or B at wavelengths λ_1 or λ_2[a];
ε_s^λ (m^2 mol^{-1})	molar absorption coefficient of the reference standard at wavelength λ, *cf.* eqn. 6[a];
λ (10^{-9} m)	wavelength used for detection;
λ_1, λ_2 (10^{-9} m)	two different wavelengths used for detection.

Compounds

1	5-[2-(Dimethylamino)ethoxy]-7-hydroxy-7*H*-benzo[*c*]fluorene N-oxide (dihydrobenflurone N-oxide);
2	5-[2-(Dimethylamino)ethoxy]-7-oxo-7*H*-benzo-[*c*]fluorene N-oxide (benflurone N-oxide);
3	5-[2-(Methylamino)ethoxy]-7-hydroxy-7*H*-benzo[*c*]fluorene (demethylated dihydrobenflurone);
4	9-Hydroxy-5-[2-(dimethylamino)ethoxy]-7-oxo-7*H*-benzo[*c*]fluorene (hydroxybenflurone);
5	5-[2-(Dimethylamino)ethoxy]-7-hydroxy-7*H*-benzo[*c*]fluorene (dihydrobenflurone);
6	5-[2-(Methylamino)ethoxy]-7-oxo-7*H*-benzo[*c*]-fluorene (demethylated benflurone);
7	5-[2-(Dimethylamino)ethoxy]-7-oxo-7*H*-benzo-[*c*]fluorene (benflurone).

NOTE ADDED IN PROOF

A method similar to that of ref. 1 was published recently[11].

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[a] Conventionally, ε^λ is expressed in 1 mol^{-1} cm^{-1} = 10^{-1} m^2 mol^{-1}.