*Journal of Chromatography*, 375 (1986) 119—128
*Biomedical Applications*
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 2910

# DIRECT QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF A NEW BENZAMIDE IN BIOLOGICAL FLUIDS

## STATISTICAL COMPARISON WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

F BRESSOLLE*, J BRES and M SNOUSSI

*Groupe de Recherche en Pharmacocinétique, Laboratoire de Chimie Analytique, Faculté de Pharmacie, Avenue Charles Flahault, 34060 Montpellier Cedex (France)*

and

R SABATIER

*Laboratoire de Physique Industrielle Pharmaceutique, Faculté de Pharmacie, Avenue Charles Flahault, 34060 Montpellier Cedex (France)*

(First received August 9th, 1985, revised manuscript received October 1st, 1985)

SUMMARY

A sensitive and selective thin-layer chromatographic (TLC) method has been developed for the determination of levels of a new benzamide, 5-(methylaminosulphonyl)-N-[(1-allyl-2-pyrrolidinyl)methyl]-2-methoxy-4-amino benzamide (RIV 2093), in urine Diazotization, followed by coupling with N-(1-naphthyl)ethylenediammonium dichloride, carried out on the thin-layer plate, has been utilized for visualization The intensity of the spots has been measured by simultaneous reflectance, with the transmittance mode at 530 nm The detection limit is 10 ng of applied material This method has been used to determine urine levels of the unchanged drug in the pharmacokinetic study of benzamide in humans after a single dose (intravenous and oral) and multiple doses (3 × 50 mg) of the drug During this study, benzamide was also determined in blood and urine by high-performance liquid chromatography, which enabled urine levels determined by the two methods to be compared by a linear structural relationship, the results were well correlated

## INTRODUCTION

In a previous paper [1], we described a sensitive and selective high-perfor-

0378-4347/86/$03 50 

mance liquid chromatographic (HPLC) method for the determination of a new benzamide, 5-(methylaminosulphonyl)-N-[(1-allyl-2-pyrrolidinyl)methyl]-2-methoxy-4-amino benzamide (I) ($pK_{a1}$ = 8 6, $pK_{a2}$ = 11 7), in biological fluids The observed detection limit (with UV detection) by that method is 12 ng/ml plasma, which corresponds to a 10-ng injection [1]. A method was required for the measurement of blood and urine levels of the unchanged drug for a study of its pharmacokinetics in twelve healthy subjects [2]

The purpose of this present paper is to describe a quantitative thin-layer chromatographic (TLC) method for the detection of compound I in urine In comparison to UV detection, an increased sensitivity and selectivity was obtained by using an in situ diazo-coupling technique after spraying the plates with Bratton-Marshall reagent [3—5] To check the validity of the method as compared to the HPLC method [1], we have used a statistical analysis approach

## EXPERIMENTAL

### *Materials and reagents*

5-(Methylaminosulphonyl)-N-[(1-allyl-2-pyrrolidinyl)methyl]-2-methoxy-4-amino benzamide (RIV 2093, I) was obtained from Delagrange (Paris, France) and was used as 0.004, 0.006, 0.01, 0 02, 0.03, 0.04 and 0.05 g/l solutions, prepared by dilution from a 1 g/l stock solution in glass-distilled water

Methanol was twice-distilled in an all-glass apparatus before use. Chloroform, ammonium hydroxide, hydrochloric acid, sodium nitrite and N-(1-naphthyl)-ethylenediammonium dichloride were all of analytical-reagent grade (Merck, Darmstadt, F R G ) Chloroform was used without further purification

Solutions of sodium nitrite, hydrochloric acid and N-(1-naphthyl)ethylenediammonium dichloride were freshly prepared in glass-distilled water and were used as 1% (w/v), 2 *M* and 0.1% (w/v) solutions, respectively

### *Thin-layer chromatography*

Separation was performed on precoated silica gel 60 glass TLC plates without fluorescent indicator (20 × 20 cm, Merck), with a layer thickness of 0.25 mm. Plates were made with 11—15 $\mu$m particles, which have a rather narrow particle-size distribution. Samples were applied to the plates using a 10-$\mu$l Hamilton syringe, calibrated at 0 1-$\mu$l intervals Spots were applied 2.5 cm from the edge and from the bottom of the plate The edge of the plate was dipped into the mobile phase to a depth of 0 8 cm

The mobile phase was chloroform—methanol—ammonium hydroxide (80 15·3). This was allowed to travel 14 cm from the point of application The plates were dried in a stream of air at 30—40°C after development and after each spray.

Spraying of the plates was carried out from a distance of ca 30 cm with a spraying device (Desaga Spray-Gun, Roucaire, France), in zig-zag movements, covering the whole of the appropriate side of the plate evenly over a period of 20 s per spray solution per plate of 20 × 20 cm

Spraying of the plates was done first with a 1% solution of sodium nitrite in water, after drying for 10 min with a hydrochloric acid solution, then, after drying, with the reagent solution

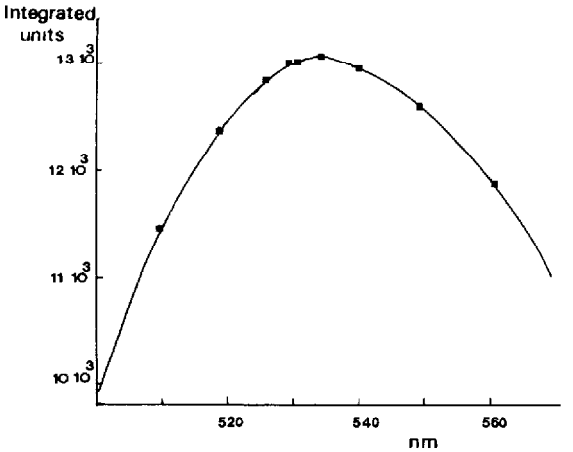


Fig 1 In situ reflectance and transmittance mode spectra of benzamide on a TLC plate (c = 40 mg/l)

Quantitation was performed with a Zeiss PMQ II spectrophotodensitometer. The signal was recorded on a chart recorder (B.B.C Goerz) and the peak areas determined with an electronic integrator (Minigrator®, Spectra-Physics, France). Measurements on the plates were carried out in the simultaneous reflectance and transmittance mode in the direction of the solvent flow with a 0 3 × 8 mm aperture slit, scanning speed 50 mm/min and paper speed 60 mm/min. When the plates containing the diazo-coupled spots were subjected to photodensitometric analysis, the maximum detector response was observed at 530 nm (Fig. 1)

*Sample preparation*

Aliquots of 3—10 μl of each urine sample were spotted directly on to the plate in 0.2-μl fractions (eleven spots per plate). Each spot was dried in a stream of air at 30—40° C

*Instrument calibration*

For calibration, 5 μl of each standard solution in urine were spotted directly This represents amounts of 20—250 ng of compound I

*Data analysis*

Peak integrated area was used as the assay parameter The relationship between spot area and analyte concentration was established on the same plate, and linear regression was applied to the data

*Statistical analysis linear structural relationship*

The standard computational methods applied in linear regression analysis assumes that only the dependent variable is affected by a random error However, it is well known that when both dependent and independent variables are affected by a random error (as in the present case), the use of this standard computational method is not appropriate, since it produces a biased estimate of the slope parameter

Many approaches have been suggested to overcome this problem [6- 8]. Different methods have been compared using a Monte Carlo simulation procedure [7], and the method of York [8] seems to be the most reliable and robust technique

In the present paper, an observed point is defined as a pair of values, $x$, $y$, where $x$ is the TLC value and $y$ the HPLC value. $x$ and $y$ are related to a straight line with intercept $\alpha$ and slope $\beta$ by

$$x = X + \epsilon \tag{1}$$

$$y = \alpha + \beta X + \delta \tag{2}$$

where $(X, \alpha + \beta X)$ is the expected value of the line and $(\epsilon, \delta)$ represents the analytical error of the observations.

Given $n$ independent observations $(x_1, y_1), \ldots (x_n, y_n)$ of $(x, y)$, the parameter $(\hat{\alpha}, \hat{\beta})$ of the straight line is estimated by minimizing the sum of squares $(SS)$:

$$SS = \sum_i \left\{ w(x_i) [x_i - \hat{x}_i]^2 + w(y_i) [y_i - \hat{y}_i]^2 \right\} \tag{3}$$

The weights, $w(x_i)$ and $w(y_i)$, are usually defined as inversely proportional to the variance of $x$ and the variance of $y$, respectively.

York [8] has shown that the least-squares solution is the root of a cubic equation in $\hat{\beta}$. The estimation, $\hat{\alpha}$, of $\alpha$ can be obtained from $\bar{y} - \hat{\beta}\bar{x}$ (where $\bar{x}$ and $\bar{y}$ are the means of $x$ and $y$); the best estimate of $\beta$ can be found by an iterative procedure. If $X$, $\epsilon$ and $\delta$ are assumed to be independent random variables, with $\epsilon$ and $\delta$ normally distributed, $(\hat{\beta}, \hat{\alpha})$ may no longer correspond to a bivariate normal distribution

Nevertheless, a simultaneous test of the null hypothesis $\alpha = 0$ and $\beta = 1$ can be approximately achieved using the $F$ test [9].

Calculations were carried out in FORTRAN using the SAS statistical analysis system (IBM 3081, Centre National Universitaire Sud de Calcul, Montpellier, France).

## RESULTS AND DISCUSSION

### *Thin-layer chromatography*

The observed $R_F$ of compound I was 0 6. The purple-red spots of the sample were stable at both room temperature and under light for 2 h (intensity decrease < 2%)

The reproducibility of the chromatographic method was determined on three solutions of compound I prepared in urine at concentrations of 6, 20 and 40 μg/ml Aliquots of 5 μl of each sample were spotted in replicate ($n$ = 10) and the spot areas at these different sample concentrations were determined The coefficients of variation were 11.7, 6.77 and 4 57%, respectively

### *Asymmetry coefficient*

The peak skew was evaluated on the chart using the asymmetry coefficient, $As$, i.e.

$$As = b/a \quad (4)$$

where $b$ is the distance after the peak maximum, and $a$ the distance before the peak maximum, both $a$ and $b$ being measured at 10% of the total peak height. For compound I, the asymmetry coefficient was found to be 1.20.

*Linearity*

In urine, the peak area varied linearly with concentration over the range given in Table I. The coefficient of the linear regression analysis ± S.D was 0.995 ± 0.004, the slope was 78.46 ± 46.3 S.D.

TABLE I

LINEAR LEAST-SQUARES REGRESSION THROUGH $x_i$ AND $y_i$

$x_i$ = Amount of benzamide spotted 20, 30, 50, 100, 150, 200 and 250 ng, $y_i$ = integrated areas corresponding to different concentrations

| Urine No | Linear regression coefficient, $r$ | Slope | Intercept | Urine No | Linear regression coefficient, $r$ | Slope | Intercept |
|---|---|---|---|---|---|---|---|
| 1 | 0 99047 | 46 88 | 1000 5 | 36 | 0 98857 | 57 56 | 1785 6 |
| 2 | 0 99166 | 83 29 | 1390 7 | 37 | 0 99202 | 74 72 | 140 8 |
| 3 | 0 99771 | 63 11 | 779 3 | 38 | 0 99838 | 80 30 | 638 8 |
| 4 | 0 99480 | 67 23 | 2514 0 | 39 | 0 98940 | 58 86 | 1977 5 |
| 5 | 0 99919 | 55 23 | 3268 6 | 40 | 0 99579 | 52 12 | 862 3 |
| 6 | 0 99047 | 46 88 | 1000 5 | 41 | 0 99864 | 71 51 | 219 2 |
| 7 | 0 99166 | 83 29 | 1390 7 | 42 | 0 99891 | 67 98 | 742 2 |
| 8 | 0 99771 | 63 11 | 779 3 | 43 | 0 98904 | 69 08 | 1074 7 |
| 9 | 0 99476 | 67 23 | 2514 0 | 44 | 0 99453 | 53 56 | 2215 0 |
| 10 | 0 99919 | 55 23 | 3268 6 | 45 | 0 99516 | 59 69 | 3018 3 |
| 11 | 0 99917 | 77 76 | 496 4 | 46 | 0 99973 | 52 47 | 4003 0 |
| 12 | 0 99600 | 37 37 | 1660 0 | 47 | 0 99589 | 52 43 | 1650 9 |
| 13 | 0 99591 | 103 52 | 3530 0 | 48 | 0 99963 | 62 25 | 888 8 |
| 14 | 0 99887 | 63 84 | 544 7 | 49 | 0 99449 | 43 37 | 84 1 |
| 15 | 0 99348 | 24 76 | 94 3 | 50 | 0 99406 | 70 40 | 1086 0 |
| 16 | 0 99110 | 88 03 | 200 5 | 51 | 0 99171 | 62 13 | 2411 2 |
| 17 | 0 99998 | 101 58 | 133 4 | 52 | 0 99964 | 80 22 | 212 7 |
| 18 | 0 98794 | 138 39 | 1435 3 | 53 | 0 99116 | 50 00 | 2698 2 |
| 19 | 0 99603 | 121 40 | 108 5 | 54 | 0 99263 | 57 57 | 1704 6 |
| 20 | 0 99778 | 65 86 | 2353 7 | 55 | 0 99643 | 69 77 | 1193 7 |
| 21 | 0 99146 | 78 23 | 1308 0 | 56 | 0 99936 | 85 31 | 542 8 |
| 22 | 0 99670 | 125 64 | 2886 0 | 57 | 0 99714 | 47 36 | 3586 3 |
| 23 | 0 99930 | 100 77 | 507 3 | 58 | 0 99379 | 63 25 | 1544 8 |
| 24 | 0 99315 | 175 34 | 1990 6 | 59 | 0 99598 | 63 34 | 1486 0 |
| 25 | 0 97475 | 85 68 | 433 1 | 60 | 0 99666 | 55 87 | 2119 6 |
| 26 | 0 98753 | 212 75 | 1177 3 | 61 | 0 98770 | 52 34 | 149 2 |
| 27 | 0 99578 | 61 89 | 1657 4 | 62 | 0 98689 | 67 66 | 2478 2 |
| 28 | 0 99838 | 68 64 | 1045 8 | 63 | 0 99246 | 83 89 | 1378 3 |
| 29 | 0 99580 | 213 45 | 2138 5 | 64 | 0 98932 | 62 32 | 3323 2 |
| 30 | 0 99999 | 77 02 | 26 8 | 65 | 0 99589 | 64 22 | 757 2 |
| 31 | 0 99941 | 325 42 | 762 8 | 66 | 0 99477 | 67 23 | 2514 0 |
| 32 | 0 99845 | 161 40 | 3644 3 | 67 | 0 99725 | 55 38 | 3320 8 |
| 33 | 0 99096 | 42 87 | 859 1 | 68 | 0 98845 | 66 41 | 2307 8 |
| 34 | 0 99519 | 31 25 | 672 12 | 69 | 0 99471 | 73 30 | 2663 9 |
| 35 | 0 99902 | 44 38 | 456 2 | | | | |

*Accuracy*

The accuracy of the method was checked on three samples of urine spiked with compound I at concentrations 5, 10 and 17.5 μg/ml Each sample was determined in replicate ($n$ = 5). Table II gives the results expressed as a percentage of the theoretical concentrations and the relative error

TABLE II

ACCURACY OF THE TLC METHOD

| Theoretical concentration (μg/ml) | Experimental concentration (μg/ml) | Mean experimental concentration ± S D (μg/ml) | Percentage dose | Relative error (%) |
|---|---|---|---|---|
| 5 0 | 5 0, 5 5, 5 0, 4 98, 5 0 | 5 096 ± 0 226 | 101 92 | +1 92 |
| 10 0 | 10 2, 10 15, 10 13, 10 08, 10 13 | 10 14 ± 0 0432 | 101 40 | +1 40 |
| 17 5 | 17 58, 17 50, 17 75, 17 63, 17 50 | 17 59 ± 0 104 | 100 51 | +0 51 |

The accuracy of the method has been tested using linear regression [10]. The linear model for regression with replicate $Y$ per $X$ is given by

$$Y_{ij} = \mu + \beta X_i + D_i + \epsilon_{ij} \tag{5}$$

where $Y_{ij}$ = $j$ experimental concentrations ($j$ = 1, , 5) of $i$ theoretical groups ($i$ = 1, 2, 3), $X_i$ = $i$ theoretical concentrations, $D_i$ = deviation of the mean, $\overline{Y}_i = \Sigma_{j=1}^{5} Y_{ij}/5$ from regression, which is assumed to have a mean of zero and a variance of $\sigma_D^2$

The results obtained and a table of regression are given in Table III.

The sum of squares ($SS$) owing to linear regression represents that portion of the $SS$ among groups that can be explained by linear regression on $X$. The $SS$ owing to deviations from regression represents residual variation around the regression line. The $SS$ within groups is the measure of the variation around each group mean.

TABLE III

TABLE WITH REGRESSION FOR ACCURACY OF THE TLC METHOD

df = Degrees of freedom, $SS$ = sum of squares, $MS$ = mean square, $F$ = $F$ value of the test

| | Source of variation | | | |
|---|---|---|---|---|
| | df | $SS$ | $MS$ | $F$ |
| Among groups | 2 | 395 223 | 197 6115 | 9285 851 |
| Linear regression | 1 | 395 2167 | 395 2167 | 62261 83 |
| Deviation from regression | 1 | 6 3477$E$ − 03 | 6 3477$E$ − 03 | 0 298 |
| Within groups | 12 | 0 2553711 | 2 1281$E$ − 02 | |
| Total | 14 | 395 4784 | | |

We first test whether the mean square ($MS$) for deviations from regression is significant by computing the variance ratio of $MS_{Y \cdot X}$ over the within-group $MS$. Since we find $F = 0.298$ ($< 1$), we accept the null hypothesis that the deviation from linear regression is 0

To test the occurrence of linear regression, we therefore tested $MS_{\hat{Y}}$ over the mean square of deviations from regression $s^2_{Y \cdot X}$ and, since $F = 62261.83$ is greater than $F_{0.05\,(1,1)} = 161.4$, we clearly reject the null hypothesis that there is no regression, or that $\beta = 0$ ($P = 0.0069$)

The equation of the linear least-squares regression is

$$\hat{Y} = 0.117 + 0.99922X \qquad (6)$$

We have used a simultaneous test for slope = 1 and intercept = 0 [9] This method gives an $F$ value of 18.825 with 2 and 1 degrees of freedom ($P$=0.1689).

Our conclusion is that the differences between theoretical and experimental concentrations are not significantly different

*Selectivity*

The method appears to be selective under the conditions described, and compound I is well separated from its expected metabolites. This selectivity was also investigated by use of two-dimensional TLC The two mobile phases were chloroform—methanol—ammonium hydroxide (80 15 3) and acetone—methanol—ammonium hydroxide (70 30 3) for a first assay, the latter mobile phase was modified to diisopropyl ether—methanol—ammonium hydroxide (50 50·3) for a second assay Compound I is very well separated and no other spot appears.

*Limit of detection*

This was evaluated as 10 ng by the amount of sample that yields a detector response equal to twice the detector noise

The TLC determination of compound I in urine is selective, reliable and sensitive (10 ng spotted) This sensitivity is equivalent to the HPLC method (10 ng in the injection) [1] Reproducibility of the TLC method through the statistical coefficient of variation was 11.7% for a low concentration of 6 μg/ml, for an equivalent level (6.25 μg/ml), the reproducibility of the HPLC method is much better, the coefficient of variation being 0.27% [1]

For measurement of plasma and red blood cell (RBC) levels of the unchanged drug for pharmacokinetic study of compound I investigated in twelve healthy subjects (six men, six women) [2], the TLC method was not used because in the concentration ranges relevant to plasma and RBC levels the analytical variances were higher for TLC. Blood levels were determined by the HPLC method [1]

During this study, blank urine was collected at regular intervals, urine concentrations were measured by HPLC and TLC methods for 48 h

The results of HPLC and TLC determination of the urine samples were compared by York's method [8]

*Results of linear structural relationship*

Fig 2 shows the scatter-gram of the two variables obtained using results from

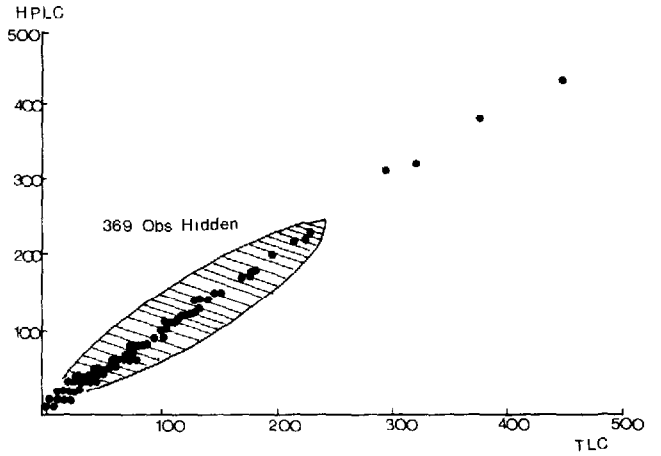


Fig 2 Parallel analysis of human samples by TLC and HPLC

444 urine samples. When using York's method [8], the range of TLC and HPLC was divided into four intervals in which the analytical variances were constant (Table IV)

The simultaneous test of identity for TLC and HPLC methods gives an $F$

TABLE IV

ANALYTICAL VARIANCES IN INTERVALS OF VARIATION FOR YORK'S METHOD

| Method | Limit of interval (mg/l) | Analytical variance | Number of replicates |
|---|---|---|---|
| HPLC | 0— 10 | 0 0590 | $n = 12$ |
| | 10— 50 | 0 81 | $n = 12$ |
| | 50—200 | 6 25 | $n = 12$ |
| | 200—400 | 2 25 | $n = 12$ |
| TLC | 0— 10 | 0 342 | $n = 20$ |
| | 10— 50 | 2 72 | $n = 20$ |
| | 50—200 | 14 10 | $n = 20$ |
| | 200—400 | 9 00 | $n = 20$ |

TABLE V

RESULTS OF YORK'S METHOD

| | |
|---|---|
| Correlation between $y$ and $x$ | 0 9980 |
| Intercept, $\hat{\alpha}$ | —0 426 |
| Slope, $\hat{\beta}$ | 0 989 |
| Correlation between $\hat{\alpha}$ and $\hat{\beta}$ | —0 4810 |
| $s^2_{\hat{\alpha}}$ | $0\ 857 \cdot 10^{-2}$ |
| $s^2_{\hat{\beta}}$ | $0\ 231 \cdot 10^{-4}$ |
| $s(\hat{\alpha},\hat{\beta})$ | $-0\ 214 \cdot 10^{-3}$ |
| $s^2_{\xi}$ | 0 7873 |
| $s^2_{\delta}$ | 6 4932 |

value of 23.77 with 2 and 442 degrees of freedom ($P < 0.0001$) Thus, the linear relationship is

$$y_{(HPLC)} = -0.426 + 0.989 \times x_{(TLC)} \quad (7)$$

The results obtained using York's method [8] are given in Table V.

The linear structural relationship shows that the results obtained from TLC are slightly overestimated in comparison with HPLC ($\hat{\alpha} = -0.426$).

In order to establish a simpler equation, we have made a test where $\alpha = -0.5$ and $\beta = 1$. This test gives an $F$ value of 2.678 ($P = 0\ 0678$), which enables us to write a new relationship, i e :

$$y_{(HPLC)} = -0\ 5 + x_{(TLC)} \quad (8)$$

Table VI gives different relative errors in some intervals of TLC concentrations

TABLE VI

RELATIVE ERRORS IN SOME INTERVALS OF TLC CONCENTRATIONS

| Limit of interval (mg/l) | Relative error (%) | Number of value (%) |
|---|---|---|
| 0— 10 | ⩾10 | 33 3 |
| 10— 50 | 10—1 | 49 1 |
| 50—200 | 1—0 25 | 15 8 |
| >200 | <0 25 | 1 8 |

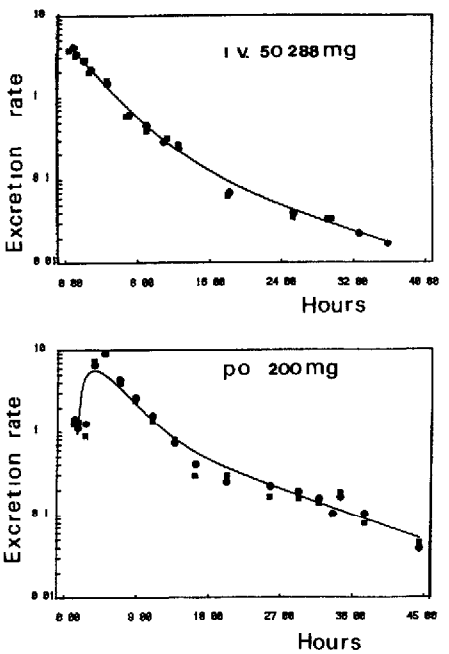


Fig 3 Benzamide urinary excretion rate following intravenous and oral administrations to a healthy subject (•) HPLC, (▪) TLC

## CONCLUSION

The TLC determination of compound I in urine is selective and sensitive. However, TLC was not used for blood sample analysis because in the concentration ranges relevant to plasma and RBC levels, the analytical variances are higher for TLC than for the HPLC method Nevertheless, for eliminated concentrations over 10 mg/l, the relative error is $< 10\%$ and the two methods are in good agreement Advantages of TLC lie in simultaneous analysis of many samples, moreover, the method is inexpensive. An example of variation of the urinary excretion rate with time (rate plot), after intravenous and oral administrations, is given in Fig. 3.

## REFERENCES


1 F Bressolle, J Bres, M Snoussi and H Laize, J Pharm Clin , Hors Série I (1985) 261
2 F Bressolle, J Bres and M Snoussi, J Chromatogr , 343 (1985) 443
3 F Bressolle, J Bres, E Fuseau and A M Siouffi, J Pharm Clin , 1 (1982) 141
4 G Huizing, A H Beckett and J Segura, J Chromatogr , 172 (1979) 227
5 J Segura, J Garcia, L Borja, E Tarrus and O H Bakke, J Pharm Pharmacol , 33 (1981) 214
6 M G Kendall and A Stuart, The Advanced Theory of Statistics, Vol 2, Griffin, London, 3rd ed , 1973
7 D S Riggs, J A Guarnieri and S Addelman, Life Sci , 22 (1978) 1305
8 D York, Can J Phys , 44 (1966) 1079
9 N Draper and H Smith, Applied Regression Analysis, Wiley, New York, 2nd ed , 1981
10 R R Sokal and F J Rohlf, Biometry, W H Freeman, New York, 2nd ed , 1981