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LIQUID CHROMATOGRAPHIC MEASUREMENT FOR PLASMA INDOMETHACIN AND ITS PRODRUG APYRAMIDE ORAL RAT AND INTRAVENOUS DOG PHARMACOKINETICS

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SUMMARY

A reversed-phase column liquid chromatographic assay is described for indomethacin (I), a non-steroidal anti-inflammatory drug, and its prodrug apyramide, an ester of I and of acetaminophen Both drugs were extracted from acetonitrile-precipitated plasma by salting them out together with acetonitrile according to the previously described solvent-demixing procedure This very simple technique proved once more to be reproducible and efficient, its recovery being similar to that of published extraction methods into non-miscible solvents The performance of the whole assay procedure was thoroughly evaluated through statistical analysis of an experimental design already proposed as a general approach to method validation A pharmacokinetic study in dogs and rats provided an illustration of the analytical method and information about the biological disposition of the ester prodrug

INTRODUCTION

Indomethacin is a widely used anti-inflammatory drug with complex pharmacokinetics in mammals [1] and in man [2] Several papers have described its reversed-phase liquid chromatographic (LC) measurement in plasma [3—10] and in urine, together with its main metabolites [11] In an attempt to reduce

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toxicity, a prodrug was created by the Laboratoires Richard apyramide, an ester of indomethacin and of acetaminophen (paracetamol) We had to devise a combined assay of the prodrug and of indomethacin, which results from its enzymatic hydrolysis, in order to study their disposition. We used the easy and reliable solvent-demixing extraction procedure, which we proposed [12] at about the same time as Mathies and Austin [13] and which we have been using since then for other drug assays [14—16] The present paper will present a statistical analysis of performance of this assay according to the experimental design that we suggested [14] and used [15, 16] for the purpose of thorough validation of assay methods The present method will be illustrated with early results on apyramide disposition following its intravenous injection to dogs and oral administration to rats

EXPERIMENTAL

Materials

Reagents and solvents were of analytical grade far-UV acetonitrile from Fisons (Loughborough, U K) through Touzart et Matignon (Paris, France), other chemicals from Rhône-Poulenc (Paris, France) Apyramide and indomethacin were gifts from Laboratoires Richard (Sauzet, France)

The chromatographic apparatus was a Spectra-Physics SP 8000 equipped with a Valco loop injector (loop volume 10 μl) and a Model 770 variable-wavelength spectrophotometric detector The column was a Radial-Pak cartridge from Waters Assoc (10 cm × 8 mm I D) filled with 10-μm μBondapak C_{18}

Male Wistar rats and male Beagle dogs were from the Centre d'Élevage of the Faculté de Pharmacie de Montpellier

Solvent-demixing extraction [12]

To 0 500 ml of plasma, 0 100 ml of 1 *M* hydrochloric acid and 0 500 ml of extraction solution were added For unknown samples, the extraction solution was a solution of internal standard in acetonitrile (2 mg/l carbamazepine), for method validation and daily standardization, it contained apyramide and indomethacin at known concentrations (spiked extraction solution) The mixture was vortex-mixed, then an excess of solid potassium chloride was added, vortex-mixed to saturation (ten brief whirls) and centrifuged (5°C, 1500 *g*, 5 min)

Chromatography

The isocratic mobile phase was a 70 30 (v/v) mixture of acetonitrile—0 01 *M* acetic acid in water, flow-rate 1 5 ml/min, detection at 250 nm, room temperature A 10-μl aliquot (loop content) of the acetonitrile supernatant was injected.

Method validation

Extraction recovery was estimated as previously described [16] $R_e = (V_eC_e)/(V_aC_a) = (V_e/V_a)\ (h_e/h_a)$ where V refers to volume, C to concentration, h to chromatographic peak height, subscript a denotes the (added) extraction

solution and subscript e denotes the extract from water or plasma. (V_e/V_a), the volumetric yield of demixing, was measured after demixing volumes ten times larger.

The validation design and suitable statistical calculations were similar to those previously described for other assays [15, 16]. Extracts were duplicated, as were measurements of each extract, allowing the variance to be split into two nested components: one related to the reproducibility of extraction and the other to the precision of chromatographic measurements. The factorial part of the design included three fixed factors: concentration (three levels corresponding to three concentrations in geometrical ratio 10), sample composition (two levels: water and plasma) and assayed drug (two levels: apyramide and indomethacin measured on the same chromatogram). Thus, the analysis of variance was performed on three concentrations × two sample compositions × two extracts per sample × two measurements per extract = 24 chromatographic runs. Measurements were ratios $y = h/h_R$ where h is drug peak height and h_R is internal standard peak height. Statistical calculations were made on the transformed values $Y = \ln y$.

Analysis of variance of the validation design was performed with an ITT 2020 microcomputer using a home-made program written in BASIC language. It included, successively: (i) a Bartlett's test of homoscedasticity on the 24 pairs of transformed Y values (12 from apyramide, 12 from indomethacin), (ii) a one-sided F-test of extract duplicates versus measurement duplicates (SE vs. SM components of variance), (iii) one-sided F-tests of each of the three factors and of each of the three interaction components versus error variance (SR, SC, SP and SRC, SRP, SCP vs. SI), (iv) regression analysis of Y versus nominal concentrations, (v) two-sided F-tests of linearity and parallelism of regression lines, (vi) a two-sided t-test of slope (deviation of the common regression coefficient from the expected value of 1).

Since conclusions of good quality were drawn from non-significance, all tests were considered significant unless $P > 0.1$ in order to reduce the β-risk (second kind) of wrong inference.

Pharmacokinetics in the dog (intravenous administration)

Equimolar doses of indomethacin (36 mg) and apyramide (50 mg), dissolved in 0.5 ml of dimethylsulphoxide (DMSO), were injected within 1.0 min into the saphenous vein of four and five dogs, respectively, whose mean weights were 11.3 kg (range 10.8–12.2 kg) and 11.8 kg (range 9.5–13.5 kg), respectively. Blood samples were collected from the contralateral vein into heparinized vials and the plasma was immediately separated and kept frozen until assayed.

Pharmacokinetics in the rat (oral route)

Equimolar doses of indomethacin (14.6 mg/kg) and of apyramide (20 mg/kg), dispersed in a 3% aqueous solution of gum-arabic, were tube-fed to two groups of fifteen rats. Three blood samples were taken from each rat, the first one at different time intervals (5 min to 8.5 h after drug administration), the second one 1 h later, the last one after either 25 or 30 h. Blood was treated as before.

RESULTS

Chromatography

Fig. 1 shows typical chromatograms of extracts from (A) water, with an extraction solution containing the internal standard (2.0 mg/l carbamazepine), apyramide (10 mg/l) and indomethacin (10 mg/l); (B) a blank plasma, with the same extraction solution, (C) a blank plasma, with an extraction solution containing only the internal standard, and (D) dog plasma following intravenous administration of apyramide

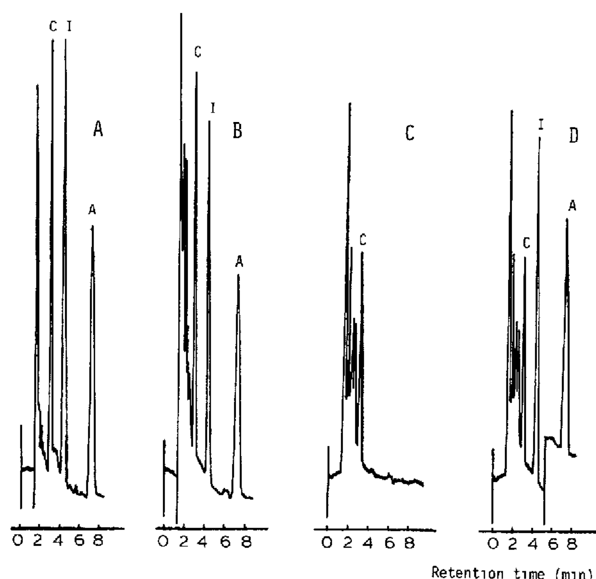

Fig 1 Chromatograms of carbamazepine (C, internal standard), indomethacin (I) and apyramide (A) (A, B) a spiked extraction solution intended for calibrations (C 2 mg/l, I and A 10 mg/ml) was used to extract water and blank plasma, respectively, (C, D) the common extraction solution containing only the internal standard was used to extract, respectively, blank plasma and a plasma sample from a dog injected with apyramide In the last chromatogram, sensitivity was halved down from 0 to 5 min in order to keep the expectedly high peak of I within the recorder range

Method validation

Table I presents extraction recoveries calculated for the internal standard and for the three concentrations of both assayed drugs The volumetric yield of demixing was 0.560 ± 0 015 [mean of five measurements ± standard error of the mean (S.E M)]

Bartlett's test, applied to the 24 pairs of measurement duplications (23 degrees of freedom), was clearly significant X^2_c = 96 336, $P < 0.001$. Nevertheless, analysis of variance of the validation design was continued (see Discussion).

Table II shows significance levels of the components of variance, along with the within-assay coefficients of variation (C.V) of measurement duplications and of extract duplications.

Estimation of between-assay precision was not included in the design It

TABLE I

EXTRACTION RECOVERY OF CARBAMAZEPINE (INTERNAL STANDARD), APYRAMIDE AND INDOMETHACIN FROM WATER AND FROM PLASMA

The spiked extraction solutions containing the compounds were chromatographed as such, after being salted out from water and from plasma Recovery was estimated for each compound as peak height after demixing, divided by peak height from the extraction solution, and multiplied by the volumetric yield of demixing Large S E M values are due to the absence of internal standardization

Compound	Concentration (mg/l)	n	Extraction recovery (mean % ± S E M)	
			From water	From plasma
Carbamazepine	2	24	75 ± 2	66 ± 2
Apyramide	20	4	86 ± 5 5	76 ± 3 5
	2	4	87 ± 2 4	80 ± 6 6
	0 2	4	95 ± 6 2	93 ± 12
Indomethacin	20	4	85 ± 5	74 ± 4
	2	4	82 ± 7	78 ± 3 5
	0 2	4	73 ± 2 3	79 ± 16

TABLE II

ANALYSIS OF VARIANCE

Sums of squares corresponding to the components of variance are symbolized as previously described [15] with the strings of suggestive letters used in the microcomputer program, namely with an S followed by T for Total variance, K for variance between all cells and I for variance within cells, R for between Rows (sample composition), C for between Columns (concentration), P for between Planes (assayed drugs), E for between all Extracts X for between Extract duplications, M for between Measurement duplications K alone is the symbol of cell total

Variance component	Sums of squares	Tests (degrees of freedom)	Comments
Within cells (nested part)	SI = ST - SK		C V = 5 73%
Measurement duplications	SM = ST - SE		C V = 5 17%
Extract duplications	SX = SE - SK		C V = 6 71%
	SX vs SM	$F(12/24) = 1\ 68$	$0\ 10<P<0\ 25$
Between cells (factorial)	SK = 1/4 ΣK^2 — correction term		
Sample composition (rows)	SR vs SI	$F(1/36)<1$	Non significant
Concentration (columns)	SC vs SI	$F(2/36) = 5000$	
Assayed drug (planes)	SP vs SI	$F(1/36) = 4\ 15$	Useless
Composition—concentration	SRC vs SI	$F(2/36) = 1\ 58$	$0\ 10<P<0\ 25$
Composition—drug	SRP vs SI	$F(1/36)<1$	Non significant
Concentration—drug	SCP vs SI	$F(2/36) = 2\ 16$	$0\ 10<P<0\ 25$
Regression	SL (common), SL_j (individual)		
Non parallelism	(ΣSL_j — SL) vs SI	$F(3/36) = 1\ 75$	$0\ 20<P<0\ 50$
Non linearity	(SC − SL) vs SI	$F(1/36) = 1\ 37$	$P = 0\ 50$
Departure of common slope from unity		$t(36) = 2\ 47$*	$0\ 01<P<0\ 02$
		$t(46) = 1\ 82$**	$0\ 05<P<0\ 10$

*Variance of the regression coefficient calculated from SI
**Variance of the regression coefficient calculated from residual variance (ST — SL)

was calculated on two dog blood samples, which were repeatedly measured in five assay sessions The results were for indomethacin (mean ± S D) sample A, 12 6 ± 0 41 mg/l, sample B, 0 146 ± 0 013 mg/l

Fig 2 shows the four bilogarithmic regressions of Y vs ln(nominal concentration) corresponding to apyramide and indomethacin extracted from water and from blank plasma Table II contains the results of tests on non-linearity, non-parallelism and departure of regressions from expected slope

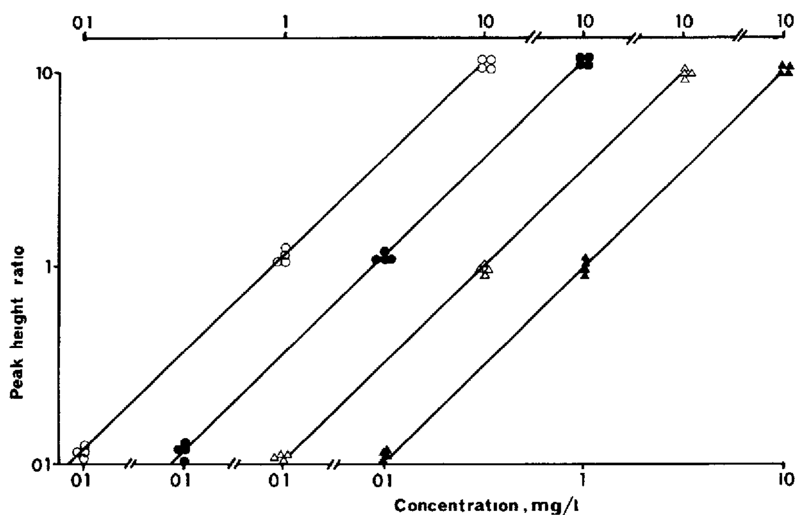

Fig 2 Bilogarithmic regressions of peak-height ratio versus nominal concentration as obtained in the validation design (○, ●) indomethacin, (△, ▲) apyramide, (○, △) plasma extracts, (●, ▲) water extracts

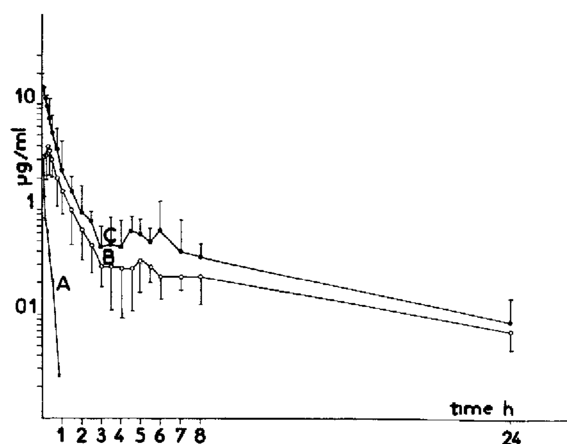

Fig 3 Dog intravenous kinetics of apyramide (A), metabolic indomethacin (B) and equimolar indomethacin (C)

Intravenous kinetics in the dog

Fig. 3 shows the evolution through time of mean plasma levels of apyramide (A), indomethacin generated from its metabolism (B), compared to indomethacin injected in equimolar dose (C)

Intravenous indomethacin levels followed a complex pattern already described in the dog [1]. A distribution phase was found again for ca 4 h with a half-time of ca. 0 5 h, as estimated between 5 and 20 min after injection Then, a slower decrease occurred during which fluctuations of mean plasma levels were obvious, even more so from individual values (not shown) Such fluctuations are ascribable to enterohepatic recycling, already described as particularly intense in the dog [1]. Plasma half-life was estimated as 7.7 h, during the elimination phase, from the values obtained 8 and 24 h after injection

Following injection of apyramide (Fig. 3A), plasma levels of the prodrug quickly decreased linearly with a half-time of ca 5 min.

Indomethacin generated from apyramide (Fig 3B) displayed at first a brief absorption phase corresponding to hydrolysis of the prodrug, which was then followed by a decrease pattern parallel to that of indomethacin injected as such, though inter-individual variations were larger and fluctuations were no longer evident. Principally, plasma levels of metabolic indomethacin were steadily lower than those of equimolar indomethacin injected per se Areas under curves, calculated between 0 and 8 h according to the trapezoidal rule, were significantly different when compared by Mann and Whitney's non-parametric test ($P < 0.05$). Their mean values (geometric means) were 21 7 mg l^{-1} h ± 10% after indomethacin, 8 68 mg l^{-1} h ± 30% after apyramide.

Oral pharmacokinetics in the rat

Apyramide could not be found in rat plasma after oral administration, even at the lowest 0 03 mg/l level that could be consistently detected in dog experiments. Fig. 4 shows the evolution through time of plasma levels of indomethacin per se and of metabolic indomethacin Pharmacokinetic rate constants were calculated by the graphical method of residuals according to a two-compartment model Absorption constants were, respectively, 0.91/h and 0 58/h, elimination constants 0 137/h and 0 097/h. Areas under the curves were tentatively calculated within the 30 h of observations: they were found in a 23:1 ratio, but this preliminary result is highly questionable Later experiments for other purposes resulted in a ratio of about 3 1. Earlier batches of apyramide probably had poor bioavailability.

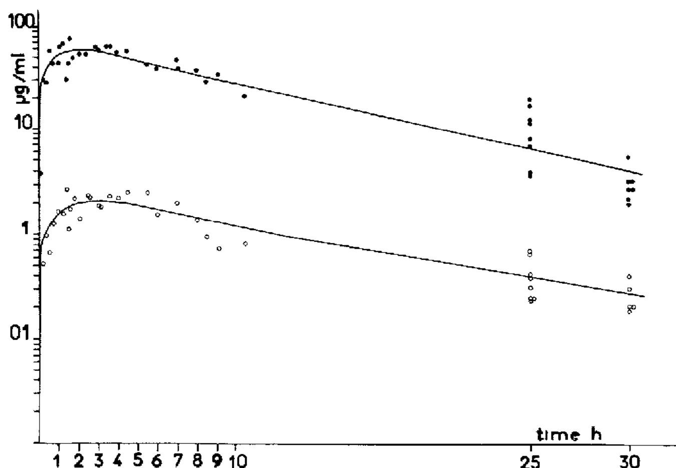

Fig 4 Rat oral kinetics of indomethacin given as such (upper curve) or as an equimolar dose of apyramide Unmetabolized apyramide was not detectable

DISCUSSION

Method validation

As described and discussed elsewhere [15, 16], the validation design used

here was intended for analysis of performance of drug assay methods. Briefly, the within-cell nested pattern of variance analysis allows the imprecision of determinations to be split into two components: one related to the measurement technique itself and the other related to sample preparation, namely an extraction technique. The factorial part of the design, together with regression analysis, gives information about the linearity of the assay relationship and about whatever factor one wants to put under study, such as the influence of plasma protein on the extraction of assayed drugs and of internal standard.

Logarithmic transformation of data allows us to: (i) homogenize variance under the hypothesis of a constant coefficient of variation, (ii) test linearity of any proportional relationship through deviations of the bilogarithmic regression slope from the expected value of 1, since if $y = a\,x$, then $\ln y = \ln x + \ln a$, (iii) estimate any coefficient of variation (C.V.) from the square root of the corresponding mean square, provided that the C.V. is small enough, i.e. residual C.V. $= s/m = \Delta\, m/m \approx dm/m = d \ln m \approx \Delta \ln m = s_{\ln}$.

In our results, the high significance of Bartlett's test ($P < 0.001$) indicated that the variance of transformed measurements was not constant, i.e. that measurements did not have a constant C.V. In fact, the logarithmic transformation overcompensates the much larger heteroscedasticity of measurements (see below under *Precision*). Such a situation would prohibit further variance analysis if it was conventionally used for the detection of significant deviations with a predefined α-risk of wrong inference. We transgressed this recommendation and carried on with the analysis because the present use of statistics in any way, though widely accepted, results in the interpretation of non-significance with an unknown β-risk of wrong inference. As already stated, in order to minimize this risk, we decided rather arbitrarily to accept significance, unless $P > 0.1$ instead of the usual 0.05; in fact, most tests had even higher P values.

Extraction reproducibility

Once more, the very easy solvent-demixing procedure proved fairly reproducible. Variance between extract replications (C.V. = 6.7%) was not significantly different from variance between measurement duplications (C.V. = 5.2%): comparison of both components resulted in $F(12/24) = 1.68$, $0.1 < P < 0.25$.

Plasma proteins do not interfere with measurements since results were not statistically different after extraction from water or from blank plasma: comparison between rows was far from significant, resulting in $F < 1$. None of the three interaction components was significant, which means that the three factors under study affected measured values independently. These results confirm, in particular, that carbamazepine is an adequate internal standard, extracted in the same ratio to both assayed drugs whatever the protein content of the sample and whatever the drug concentration.

Recoveries of apyramide and of indomethacin were similar. The latter was comparable to those that have been published with non-miscible solvents: 68% [4] with dichloroethane, 66% [6] and 85% [5] with dichloromethane, 82% (double extraction) [8] and 94–100% [7] with ethyl acetate, 90% [9], 75% [10] and 85% [3] with diethyl ether.

Assay relationship

Regression departed neither from parallelism ($0.2 < P < 0.5$) nor from linearity ($P \approx 0.5$). However, common regression departed significantly from the expected unit slope From individual values of the four regression coefficients, it appeared that the fault lay with apyramide (from water: b = 0 975, from plasma: b = 0 985) rather than with indomethacin (0 995 and 1 001, respectively).

Precision

Since between-extract variance, although larger (C.V. = 6 7%), did not differ significantly from within-extract variance (C V = 5 2%), both were considered as estimates of within-assay variance and their pooled value (within-cell variance) was taken as error variance. The corresponding C V was 6%, as in our previous chromatographic assays of other drugs [14—16] Separate calculations for indomethacin ranged from 7 9% at the lowest concentration (0 2 mg/l) to 1 6% at the highest (20 mg/l) The present method thus has within-assay precision comparable to other recently published chromatographic methods 13.6—8.8% between 0 25 and 0 5 mg/l [8], 4% between 0 25 and 25 mg/l [7], 3% between 0 25 and 8 mg/l [9], 5 and 7% at, respectively, 0 5 and 0.25 mg/l [10]

Our separate values confirm that logarithmic variance was not constant, the variance ratio between extreme concentrations amounting to $(7.9/1.6)^2 = 24.4$ However, the corresponding variance ratio of original data would have been $(1.6 \times 20/7.9 \times 0.2)^2 = 410$ Thus, logarithmic transformation does not suppress heteroscedasticity, but does considerably lower it

Whereas within-assay precision reflects the intrinsic performance of the method, between-assay precision mainly reflects the reproducibility of daily standardizations. In our pharmacokinetic experiments, all the samples from the same pharmacokinetic run were assayed in the same assay run, our results were obtained with the same batches of spiked extraction solutions and with three-point standard curves

Intravenous kinetics in the dog

Results presented in Fig 3A show the very rapid decrease of plasma apyramide according to linear kinetics, with a half-life of ca. 5 min. Such a fast disappearance is hardly explainable by hepatic or renal clearance. The matter is either ester hydrolysis by plasma esterases already observed in vitro [17] or sequestration into a deep compartment endowed with a high transfer constant from plasma, or more probably both as will be discussed

As already noted under Results, the low rate of elimination of intravenous indomethacin from the 3rd to the 8th h (Fig 3C) is best explained by entero-hepatic recycling [1], the 7 7-h plasma half-life observed beyond the 8th h is in accordance with the 5—12 h observed by the authors (as calculated from their data in Table 3) The more than 50% deficiency of metabolic indomethacin in plasma compared to equimolar indomethacin per se is best explained by tissue sequestration of apyramide and/or sequestration peculiar to metabolic indomethacin. Indeed, sequestration of indomethacin per se, which has been suggested in man [2], would have occurred in both cases and consequently

could not explain the deficiency after intravenous apyramide compared with after intravenous indomethacin.

In the hypothesis of reversible sequestration of apyramide, it must be expected that the area under the concentration versus time curve of metabolic indomethacin from zero to infinite time would come up to the area under the curve of indomethacin injected per se, which would require a slower decrease of late plasma levels of metabolic indomethacin A verification of this hypothesis through single-dose pharmacokinetic study over a long period would probably lack precision However, a slight favourable evidence in the present work is that plasma half-life estimated between 8 and 24 h was about twice as long after apyramide as after indomethacin (15 9 h instead of 7.7 h)

The other hypothesis, namely sequestration peculiar to metabolic indomethacin and therefore irreversible or at least stronger than that of indomethacin as such, cannot be excluded a priori It is well known that acetyl anions generated by esteratic hydrolysis of aspirin are somehow activated since they are able to acetylate various tissue proteins that acetate cannot Whether such a chemical activation occurs for other anions is not known to us, but it could account for irreversible sequestration of metabolic indomethacin through an analogous acylation of tissue constituents Of course, even if sequestration was on plasma proteins, the irreversibly bound fraction would escape extraction and assay. However, such a phenomenon could hardly account for the observed deficiency of about half the injected dose without resulting in toxic consequences that do not appear to have been observed during chronic toxicity studies of apyramide [18]

REFERENCES

1 H B Hucker, A G Zacchei, S V Cox, D A Brodie and N H R Cantwell, J Pharmacol Exp Ther , 153 (1966) 237
2 D E Duggan, A F Hogans, K C Kwan and F G McMahon, J Pharmacol Exp Ther , 181 (1972) 563
3 G G Skellern and E G Salole, J Chromatogr , 114 (1975) 483
4 S J Soldin and T Gero, Clin Chem , 25 (1979) 589
5 C P Terweij-Groen, S Heemstra and J C Kraak, J Chromatogr , 181 (1980) 385
6 J L Shimek, N G S Rao and S K Wahba Khalil, J Liq Chromatogr , 4 (1981) 1987
7 M S Bernstein and M A Evans, J Chromatogr , 229 (1982) 179
8 A Astier and B Renat, J Chromatogr , 233 (1982) 279
9 J K Cooper, G McKay, E M Hawes and K K Midha, J Chromatogr , 233 (1982) 289
10 C -N Ou and V L Frawley, Clin Chem , 30 (1984) 898
11 P C Smith and L Z Benet, J Chromatogr , 306 (1984) 315
12 R Alric, Analusis, 9 (1981) 289
13 J C Mathies and M A Austin, Clin Chem , 26 (1980) 1760
14 R Alric, M Cociglio, J P Blayac and R Puech, J Chromatogr , 224 (1981) 289
15 M Cociglio, D Sauvaire and R Alric, J Chromatogr , 307 (1984) 351
16 R Alric, L Arce-Corrales, J P Blayac and R Puech, Methods Find Exp Clin Pharmacol , 6 (1984) 353
17 J L Chanal, unpublished results
18 J J Serrano, unpublished results