Journal of Chromatography, 583 (1992) 137–143
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6575

Use of hydrophilic interaction chromatography for the study of tyrosine protein kinase specificity

Jean A. Boutin, Anne-Pascale Ernould, Gilles Ferry and Annie Genton

Laboratoire de Biochimie Moléculaire, Département de Cancérologie Expérimentale, Institut de Recherches Servier, 11 Rue des Molineaux, 92150 Suresnes (France)

Andrew J. Alpert

PolyLC Inc., 9151 Rumsey Road, Suite 180, Columbia, MD 21045 (USA)

(First received June 23rd, 1992; revised manuscript received September 9th, 1992)

ABSTRACT

A new HPLC method has been developed to assay tyrosine protein kinase activity. Using hydrophilic interaction chromatography, it is possible to resolve the four components of the incubation medium: substrate peptide, [^{32}P]phosphorylated peptide, unreacted [γ-^{32}P]ATP, and ^{32}P-labelled inorganic phosphate. ATP interacts so strongly with the stationary phase material that it can be removed selectively from the incubation medium with solid-phase extraction cartridges packed with the same type of material. The three remaining components of interest can then be resolved by reversed-phase or hydrophilic interaction HPLC. This procedure permits the evaluation of almost every type of peptide as a substrate of tyrosine protein kinase.

INTRODUCTION

Since the description of the first procedure of a tyrosine protein kinase (TPK) [1], the different molecular forms have been intensively studied in order to ascertain their roles in cellular growth and differentiation. Over 30 individual TPKs have been described [2]. The elucidation of their biochemical significance requires the determination of their substrate specificities. However, most published work concerns their molecular biology [3] rather than their substrate specificity [4–8]. This situation may reflect the inadequacy of the techniques available for the measurement of TPK activity. The use of phosphocellulose paper [9] is limited to arginine-containing peptides [10]. The method of Braun *et al.* [11] seems to be of more general utility [5–8], albeit time-consuming. An enzyme-linked immunosorbent assay (ELISA) [12] requires that peptides be coated on microwell plates.

Ferry *et al.* [13] introduced a method based on RP-HPLC. This method works well with markedly hydrophobic peptides. However, acidic residues are of major importance in the recognition of many substrates by TPKs, as with minigastrin (LEEEEEAYGWMDF, using the single letter code for amino acids). Substrates with such residues are too hydrophilic for the RP-HPLC method. Phosphorylation of the peptides increases their hydrophilicity still further. Accordingly, we have applied the new technique of hydrophilic interaction chromatography (HILIC) [14] to this

Correspondence to: J. A. Boutin, Institut de Recherches Servier, 11 Rue des Moulineaux, 92150 Suresnes, France.

0378-4347/92/$05.00

problem. Solutes elute in this mode in order of increasing hydrophilicity, using decreasing gradients of organic solvent, increasing gradients of salt, or both together. This development permitted us to pursue two objectives in this study: (1) to assess the substrate specificity, using any substrate, of a particular TPK; (2) exploiting such data to design high-affinity substrates as potential specific inhibitors. This study presents data obtained from three peptides based on the copolymer poly(Glu · Na,Tyr) 4:1, widely used as a substrate for TPKs. These peptides included EY, EYE, and EEYEE, as well as minigastrin derivatives. Angiotensin II was used as a standard control.

EXPERIMENTAL

Biological source

A TPK was partially purified [15,16] from HL-60, a human promyelocytic cell line. The activity was *ca.* 2300 times greater than that in the crude extract.

Peptides

All peptides were synthesized by NeoSystem (Strasbourg, France). They were individually sequenced and analysed by the manufacturer, and were over 95% pure by HPLC. Angiotensin II was from NeoSystem or Sigma (St. Louis, MO, USA). HILIC columns and solid-phase extraction (SPE) cartridges packed with PolyHydroxyethyl Aspartamide (PolyLC, Columbia, MD, USA) were provided by C.I.L. (Ste-Foy-la-Grande, France). All chemicals were commercially obtained, in the highest purity available.

Assay

The regular TPK assay uses angiotensin II as substrate and [γ-^{32}P]ATP as phosphate donor. It has been described elsewhere [12], but has been modified for use with HILIC. Thus, a 100-μl final volume contains 40 μl of HMM buffer [20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–NaOH (pH 7.4), 5 m*M* $MgCl_2$, 5 m*M* $MnCl_2$], 10 μl of angiotensin II (10 mg/ml, in water), 10 μl of ATP [100 μ*M*, containing 30 μCi/ml [γ-^{32}P]ATP (Amersham, UK)], 20 μl of saturate NaCl solution, and 20 μl (2 μg) of the partially purified enzyme preparation, with a specific activity of 2.5 nmol/min/mg protein. Incubations were performed for 30 min at 30°C and were stopped by the addition of 500 μl of either 50% trichloroacetic acid (RP-HPLC) or 20 m*M* triethylamine phosphate (TEAP) (pH 2.8)–acetonitrile (50:50) (HILIC). An aliquot of 30 μl of the resulting fluid was injected for HPLC analysis.

When peptides other than angiotensin II were chosen, stock solutions (10 mg/ml) were prepared.

Instrumentation

The HPLC system consisted of a Waters 840 workstation, including a 380 PC computer (DEC), a solvent-delivery system (two Model 510 pumps, one linked to an automatic four-way valve selector), a Model 490 UV-VIS absorbance detector, and a WISP Model 712, all obtained from Waters (Milford, MA, USA), and a Model LB-504 on-line radioactivity monitor from Berthold (Wildbad, Germany) directly coupled to the data acquisition unit. Detection of ^{32}P (Cerenkov effect) was at efficiency of *ca.* 35%, as determined by us and in agreement with the manufacturer's specifications. The analysis cell in the monitor had a volume of 1 ml, to enhance the sensitivy of detection. As a consequence, the signals showed an increased peak width.

RP-HPLC

Routine assay. This was developed previously [13] and is used routinely in our laboratory. The procedure is based on the use of tetrabutylammonium phosphate as a counterion.

Assay after prepurification by use of SPE. Simplification of the RP-HPLC assay was achieved after passing the incubation fluid through HILIC-SPE cartridges (see below). A Nucleosil C_{18} column, 100 × 4.6 mm I.D. (7 μm particle size, 300 Å pore diameter) from Macherey–Nagel (Düren, Germany) was used with a 40-min linear gradient between solution A (0.05% triethylamine + 0.1% trifluoroacetic acid (TFA), aq.) and solution B (0.1% TFA in 100% acetonitrile). The flow-rate was 2.0 ml/min.

Hydrophilic interaction chromatography

Procedure without prepurification by SPE. A PolyHydroxyethyl A column (200 × 4.6 mm I.D.; 5 μm particle size, 300 Å pore size) was rinsed initially with 20 ml of water followed by 60 ml of 800 m*M* TEAP (pH 2.8). It was then equilibrated with 30 ml of the starting solution A, consisting of 4 m*M* TEAP (pH 2.8), containing 90% acetonitrile. The great affinity of ATP for the column matrix necessitated a complex gradient (hereafter referred to as the regular gradient): 0–5 min, 100% solution A; 5–20 min, linear gradient to 100% of 10 m*M* aq. TEAP (pH 2.8); 20–25 min, 40 m*M* aq. TEAP (pH 2.8); 25–40 min, 800 m*M* aq. TEAP (pH 2.8); 40–50 min, water. Reequilibration was carried out for *ca.* 10–15 min with 100% solution A. The total analysis time was 70 min. The flow-rate was 1.5 ml/min.

Application of the SPE procedure. Disposable SPE cartridges (10 × 4.6 mm I.D.) of PolyHydroxyethyl A were used in a holder with luer fittings. Prior to HPLC analysis, incubation media were subjected to SPE as follows: (a) preequilibration of the cartridges (via syringe) with 3 ml of 20 m*M* TEAP (pH 2.8) containing 50% acetonitrile; (b) passage (via syringe) of the assay medium (600 μl) through the cartridge; (c) rinsing of the cartridge with 1 ml of 20 m*M* aq. TEAP (pH 2.8); (d) elution of ATP with 2 ml of 800 m*M* aq. TEAP (pH 2.8).

All eluates were individually collected in microfuge tubes. The tubes were counted by measurement of Cerenkov radiation in a Beckman LS-1800 radioactivity counter. A sample aliquot of 30 μl was then analysed using a gradient for HILIC shorter than that described above: 0–5 min, 100% solution A; 5–20 min, linear gradient to 100% of 10 m*M* aq. TEAP (pH 2.8). The flow-rate was 1.5 ml/min, and the total running time was 30 min, including reequilibration to the initial conditions.

RESULTS

Peptide phosphorylation experiments

In a standard TPK assay, five components are present or potentially generated: peptide substrate (I); ^{32}P-labeled phosphorylated product (II); unreacted [γ-^{32}P]ATP (III); ADP (IV); and inorganic radiolabeled phosphate (^{32}Pi) (V). Assessment of substrate specificity requires separation of II from III and V. Some of the peptides studied here as substrates were based on the sequence of minigastrin, with single residues omitted sequentially from either C- or N-termini. Using the HILIC mode with a decreasing acetonitrile gradient, such hydrophilic peptides typically elute around 55–60% acetonitrile (Fig. 1). However, ATP was retained so strongly in this mode that its elution required a completely aqueous solution containing 800 m*M* TEAP. Such strong solute–matrix interactions in this mode have also been noted with other hydrophilic polyelectrolytes such as RGD peptides and aminooligoglycosides [17]. Nonetheless, the method proved to be feasible for studying the phosphorylation at Tyr of almost any type of peptide. Fig. 2 shows the profiles obtained by HILIC using angiotensin II as substrate. Table I summarizes the data obtained with minigastrin-derived peptides.

A non-negigible amount of ^{32}Pi was observed during the analysis. This was present as a contaminant in the commercially available [γ-^{32}P]ATP solutions from both Amersham and New England Nuclear (NEN), representing 3–5% of the total radioactivity. Positive identification of this peak in our system was performed by injecting ^{32}Pi (NEN). When [α-^{32}P]ATP was used in the assay, no angiotensin II was labeled with ^{32}P; the major labeled peak coeluted with [γ-^{32}P]ATP, while the level of angiotensin II phosphorylated

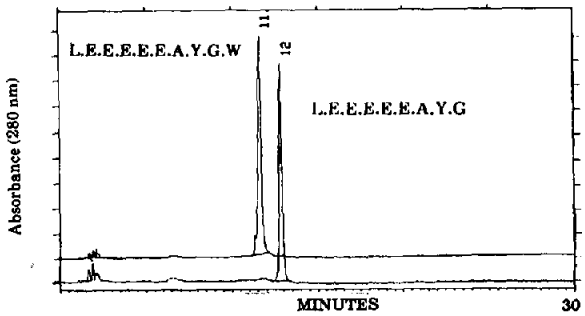

Fig. 1. HILIC of minigastrin derivatives (shortened gradient: see Experimental). LEEEEEAYG (top) elutes at 12 min; LEEEEEAYGW (bottom) at 11 min.

TABLE I

PHOSPORYLATION OF MINIGASTRIN DERIVATIVES BY A PARTIALLY PURIFIED TPK FROM HL-60

Peptide	Retention time of phosphorylated peptide (min)	Rate of phosphorylation (rel. %)[a]
DRVYIHPF[b]	–	100
LEEEEEAYG	17.8	133
LEEEEEAYGW	14.6	191
LEEEEEAYGWM	9.0	300
LEEEEEAYGWMD	8.2	86
LEEEEEAYGWMDF[c]	7.7	310
EEEEEAYGWMDF	n.d.[d]	0
EEEEAYGWMDF	9.1	280
EEEAYGWMDF	8.8	290

[a] The phosphorylation reactions were conducted as described in Experimental, and the analysis was performed by HILIC (regular gradient without SPE). 100% represents the reaction rate with angiotensin II as substrate (2.5 nmol/min/mg protein).
[b] Angiotensin II.
[c] Minigastrin.
[d] n.d. = Not detectable.

with unlabeled phosphate was far below our UV detection limits (data not shown). ^{32}P-Phosphorylated angiotensin II was poorly separated from the peak of ^{32}Pi (Fig. 2). This is the major problem encountered so far with this method. It might be overcome by modifying the gradient slope for peptides that elute so close to Pi, or by using a C_{18} column (see below).

Use of SPE prepurification

When assay mixtures were passed through SPE cartridges of PolyHydroxyethyl A (see Experimental), with collection of three eluate fractions, most of the radioactivity was eluted by 800 m*M* TEAP (pH 2.8). A minor amount of radioactivity is still retained by the cartridge after this step. Fig. 3 shows the radioactivity profiles (HILIC) of the three eluates as well as a control, which consisted of the genuine (non-treated) assay medium. Evidently, ^{32}Pi and phosphorylated peptide eluted in eluates 1 and 2 with minor amounts of ATP. More than 95% of the labeled ATP eluted in eluate 3. Thus, this technique is suitable for performing assays of various peptides as substrates

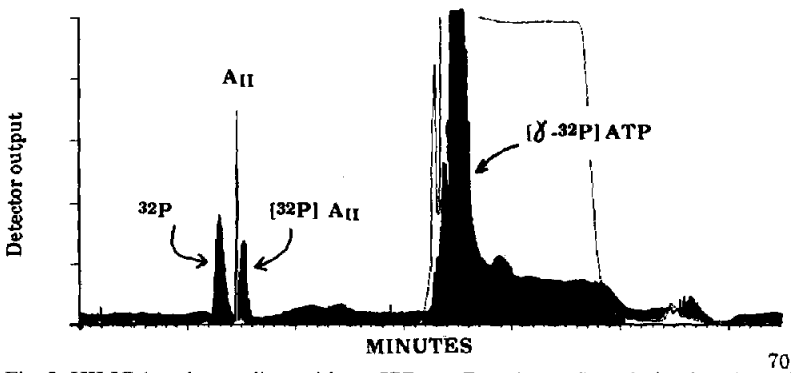

Fig. 2. HILIC (regular gradient without SPE; see Experimental) analysis of angiotensin II phosphorylation by TPK from HL-60. A 30-μl aliquot of incubation medium was analysed. The black profile shows the radioactivity monitor (2500 mV full scale output). The open profile shows the absorbance at 280 nm (0.1 a.u.f.s.).

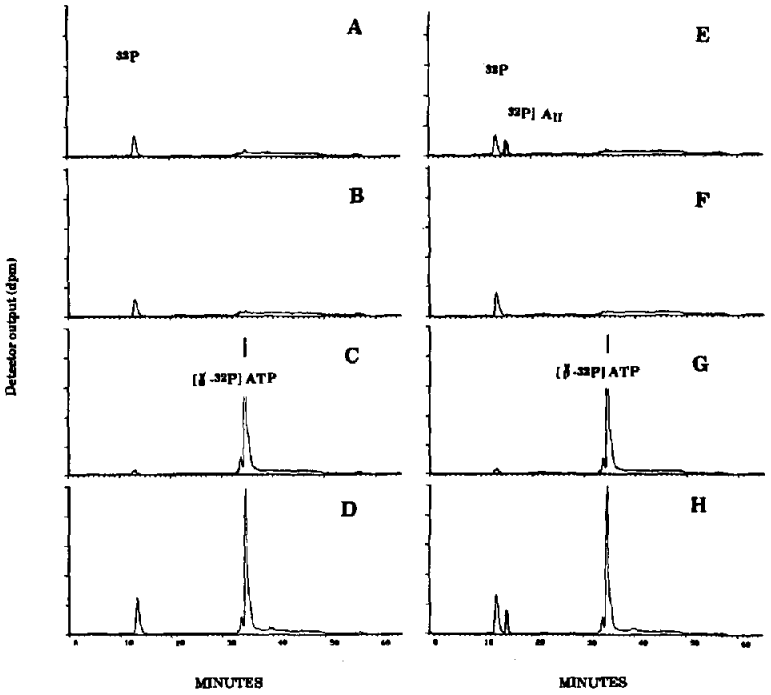

Fig. 3. Effect of SPE cartridge filtration on the products from phosphorylation of angiotensin II by TPK from HL-60. Profiles A, B, C and D: no angiotensin II (blanks). Profiles E, F, G and H: angiotensin II present as substrate. Analysis, HILIC (regular gradient); detection, radioactivity monitor. Profiles A and E: eluate from initial passage of sample through cartridge. Profiles B and F: eluate from 20 m*M* TEAP wash. Profiles C and G: eluate from the rinse with 800 m*M* TEAP. Profiles D and H: whole incubation medium (control).

of TPKs. Since it is difficult to predict whether the product will be in eluate 1 or eluate 2, both should be analysed, either individually or together.

Fig. 4 presents HPLC profiles of assays of three acidic peptides as yet untried as substrates of this type of enzyme activity: EY, EYE, and EEYEE. It is clear that their phosphorylation could not be followed using the RP-HPLC method previously developed [12], owing to their high polarity and the incomplete separation of the phosphorylated products from unreacted ATP. In contrast, the HILIC method shows phosphorylated products cleanly separated from both ^{32}Pi and ATP (after prepurification via SPE). The peptide EEYEE was superior to angiotensin II as a substrate, and EYE was comparable. EY was not recognized as a substrate by this TPK.

If ATP is extracted with an HILIC SPE cartridge, then the feasibility of the RP-HPLC increases in most cases. This is shown in Fig. 5, using a Nucleosil C_{18} column. In the case of angiotensin II, the problem of poor separation of phosphorylated peptides from ^{32}Pi has been overcome by this technique. ^{32}Pi elutes in the void whereas phosphorylated angiotensin II is well retained.

DISCUSSION

HILIC seems to be an effective tool for the assay of TPK substrates, and is applicable to a variety of peptides with a wide range of polarity, including those with extremely high polarity. The use of the SPE procedure permits extensive removal of unreacted ATP and thus is less time-consuming than the method of Braun *et al.* [11]. Removal of the ATP by SPE may make RP-HPLC practical in marginal cases. Nevertheless, the use of HILIC without prepurification by SPE

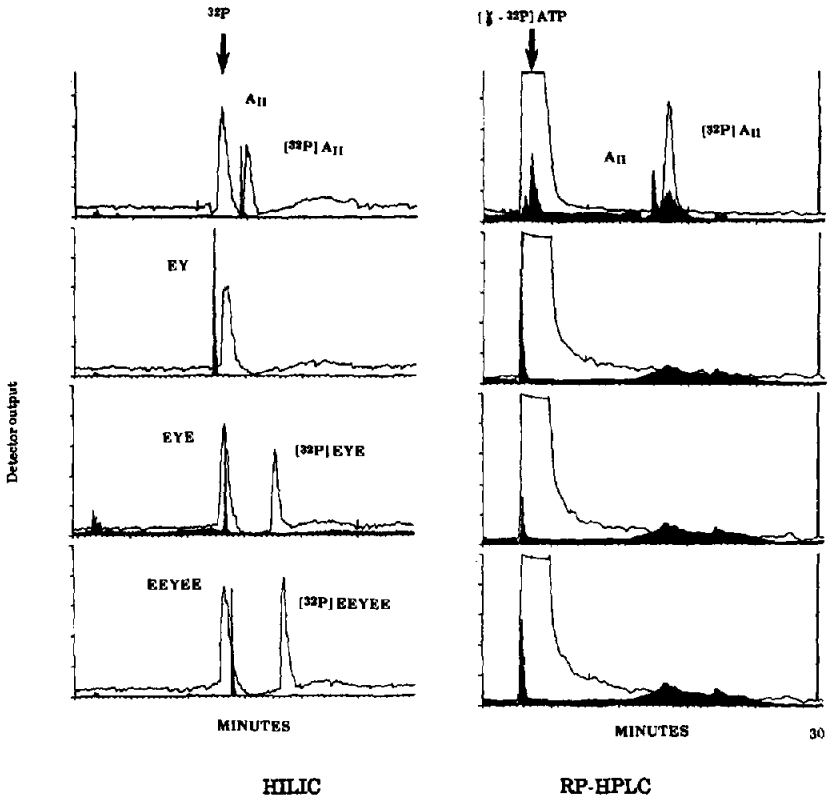

Fig. 4. Comparison of HILIC (shortened gradient with prepurification by SPE) and RP-HPLC for analysis of the phosphorylation of peptide substrates by TPK from HL-60. Identical incubations were performed in parallel and were stopped as noted in Experimental. In each case, 30-μl aliquots were analysed. The open profiles show the radioactivity monitor, and the black profiles the absorbance at 280 nm. Radioactivity detector output was 1000 mV (HILIC) or 2500 mV (RP-HPLC) full scale output. In the HILIC profiles, the peak of radioactivity eluting at 13 min is ^{32}Pi; this coincides with the elution of unlabeled peptide EYE.

has been successfully applied to the phosphorylation of more than 100 peptides of different sequences [18]. No major limitations have been observed so far. Furthermore, the great affinity of ATP for PolyHydroxyethyl A suggests the possibility of several interesting new methods, such as the measurement of ATPase activity [19] and the assay of TPK activity using proteins or copolymers as substrates.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. B. Serkiz for his HPLC expertise, and to Dr. J. F. Prost for his constant interest in our research.

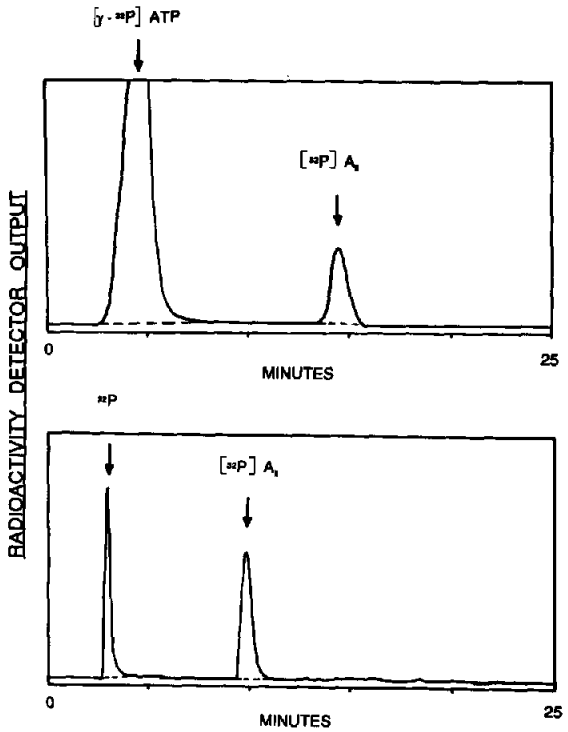

Fig. 5. Effect of SPE filtration on the analysis of angiotensin II phosphorylation medium by RP-HPLC. Top: whole medium, reaction stopped as for RP-HPLC analysis; analysis conditions as in ref. 13. Bottom: reaction stopped as for HILIC analysis, processed with a HILIC-SPE cartridge, and the eluate (initial eluate and 20 m*M* TEAP wash) analysed by RP-HPLC, using the conditions described under Experimental, with a Nucleosil C_{18} column. Detection, radioactivity monitor; 2500 mV full scale (top) or 1000 mV full scale (bottom). The shift in retention times between the top and bottom traces are due to different gradient slopes (see Experimental).

REFERENCES

1 R. L. Erickson, M. S. Collett, E. Erickson and A. F. Purchio, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 6260.
2 T. Hunter, *Cell*, 50 (1987) 823.
3 S. K. Hanks, A. M. Quin and T. Hunter, *Science (Washington, D.C.)*, 241 (1988) 42.
4 R. L. Geahlen and M. L. Harrison, in B. E. Kemp (Editor), *Peptides and Protein Phosphorylation*, CRC Press, Boca Raton, FL, 1990, p. 239.
5 A. M. Brunati and L. A. Pinna, *Eur. J. Biochem.*, 172 (1988) 451.
6 C. Cola, A. M. Brunati, G. Borin, P. Ruzza, A. Calderan, R. De Castiglione and L. A. Pinna, *Biochim. Biophys. Acta*, 1012 (1989) 191.
7 A. M. Brunati, F. Marchiori, P. Ruzza, A. Calderan, G. Borin and L. A. Pinna, *FEBS Lett.*, 254 (1989) 145.
8 P. Agostinis, J. Goris, L. A. Pinna and W. Merlevede, *Eur. J. Biochem.*, 189 (1990) 235.
9 D. B. Glass, R. A. Masaracchia, J. R. Feramisco and E. G. Krebs, *Anal. Biochem..*, 87 (1978) 566.
10 J. L. Pike, *Methods Enzymol.*, 146 (1987) 353.
11 S. Braun, M. Abdel Ghany and E. Racker, *Anal. Biochem.*, 135 (1983) 369.
12 I. Lazaro, M. Gonzalez, G. Roy, L. M. Villar and P. Gonzalez-Porque, *Anal. Biochem.*, 192 (1991) 257.
13 G. Ferry, A. P. Ernould, A. Genton and J. A. Boutin, *Anal. Biochem.*, 190 (1990) 32.
14 A. J. Alpert, *J. Chromatogr.*, 499 (1990) 177.
15 J. A. Boutin, A. P. Ernould, A. Genton and C. A. Cudennec, *Biochem. Biophys. Res. Commun.*, 160 (1989) 1203.
16 A. P. Ernould, G. Ferry, A. Genton, C. A. Cudennec and J. A. Boutin, *Anticancer Res.*, 10 (1990) 197.
17 A. J. Alpert, P. C. Andrews, J. A. Boutin, G. Ferry, P. Kostel and C. Mizzen, in preparation.
18 A. P. Ernould, G. Ferry, J. M. Barret, A. Genton and J. A. Boutin, submitted for publication.
19 J. M. Barret, A. P. Ernould, M. H. Rouillon, G. Ferry, A. Genton and J. A. Boutin, *Chem.-Biol. Interact.*, (1992) in press.