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Some strategies for improving specificity and sensitivity in the analysis of anti-cancer drugs

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Abstract: Some approaches are discussed for introducing specificity and sensitivity into analytical methods for anti-tumour agents which include a liquid chromatographic step. Various modes of HPLC have been exploited to monitor these drugs specifically and at therapeutically low levels. The use of column switching technology and chemical derivatization techniques to enhance both specificity and sensitivity are discussed. Multiple columns (linked through switching valves) containing packings exhibiting different affinities for the analytes cisplatin and riboxamide provide (a) a high degree of selectivity with convenient analysis times, (b) the opportunity for preconcentration of analytes, (c) improved longevity of analytical columns, (d) a solution to the 'general elution problem', and (e) allow direct application of biological fluid to the HPLC system. The use of chemical derivatization techniques (pre- and post-column) to achieve improved sensitivity and altered chromatographic and chemical properties of these and other anti-tumour agents (galactitol, tamoxifen, emetine) is also described. The high chemical reactivity of many anti-tumour agents often requires their rapid derivatization after a biological sample is drawn to prevent chemical degradation in the sample vial. The use of chemical and photochemical derivatization techniques combined with spectrophotometric, fluorometric and voltammetric detectors illustrates the power and utility of derivatization technology in trace drug analysis.

Keywords: *HPLC detectors; pre-column derivatization; post-column derivatization; cisplatin; tamoxifen; dianhydrogalactitol.*

Introduction

Anti-cancer drugs, unlike members of most other drug classes, are highly toxic and are often administered at doses approaching those producing life-threatening toxicities. The onset and intensity of both chemotherapeutic and toxic responses are usually dose-related. Thus, the ability to monitor such drugs and their biologically active metabolites in clinical situations is important for their optimal clinical utilization, as well as for patient safety.

Monitoring any drug in a biological system is complicated by the heterogeneity of the matrix in which the analyte(s) reside(s). Matrix composition is unknown and variable, differing from patient to patient and showing intrapatient variability influenced by diet, emotional and physical status, and secondary agents administered with the drug of

interest. The drug must be separated from these matrix components, which are often present at concentration orders of magnitudes greater than the analyte itself.

Many drugs, but particularly anti-tumour agents, are of limited stability in biological systems. Some undergo facile chemical degradation (alkylating agents); others require enzymatic transformation to produce the active species; and still others undergo extensive metabolic degradation. Thus, methodology must offer the specificity adequate to separate the drug from matrix components, and also be capable of differentiating among drug-derived species (e.g. metabolites, degradation products) which differ only subtly from one another. Consequently, much effort is needed to introduce adequate specificity into the methodologies for the analysis of antineoplastic agents.

Generally, the analytical procedure can be separated into four stages: (a) gross separation of analytes from the biological matrix, (b) high efficiency separation of compound(s) of interest from their (bio)degradation products and from endogenous contaminants, (c) detection and, (d) signal processing and data manipulation. Specificity can be introduced into the methodology at any of these stages, but many clinical drug analyses require a chromatographic step to provide the necessary selectivity. In this article, several approaches to selectivity in analytical methodologies will be discussed. The discussion will be restricted to methods incorporating a liquid chromatographic (HPLC) step, and will not attempt to review other approaches.

Separation of Drugs from Biological Matrices

The separation of drugs from biological fluids is commonly accomplished through liquid–liquid or liquid–solid extraction in the normal or ion-pair mode. These approaches have also been applied to the isolation of analytes from tissue, following tissue digestion by chemical, thermal or enzymatic means.

Liquid–solid extraction generally offers a more efficient (i.e. greater number of theoretical plates) means for separation than that provided by the corresponding liquid–liquid technique. The solid phase can be varied (to include ion-exchange and reversed phase materials, adsorbents, size exclusion gels, etc.) and the eluent can be tailor-made to provide broad separation possibilities. In addition, compounds whose physical properties preclude their isolation by liquid–liquid techniques (i.e. poor partition characteristics into water immiscible solvents) can often be handled readily by liquid–solid extraction.

Liquid–solid extraction has often been avoided in clinical studies, however, because (a) there may be loss of compound to the stationary phase, and (b) it may be time consuming and labour intensive. However, with HPLC systems, the column used for sample preparation can be 'piggy-backed' on to the analytical column using a column switching configuration as illustrated in Fig. 1. Two columns containing packings with different column selectivities are connected in series through a six-port valve. Compounds partially resolved on the first column may be switched to a second column for further separation. Such technology has been successfully applied to the analysis of the anti-tumour agents cisplatin **1** [1] and riboxamide **2** [2].

In both cases, untreated biological samples (plasma or urine) containing **1** or **2** are applied to a column containing silica gel onto which is adsorbed the cationic surfactant hexadecyltrimethylammonium bromide (HTAB). Using an aqueous mobile phase, initial eluent is directed to waste: under microprocessor control and at the point immediately prior to analyte elution, a heart-cut of the eluent containing **1** or **2** is

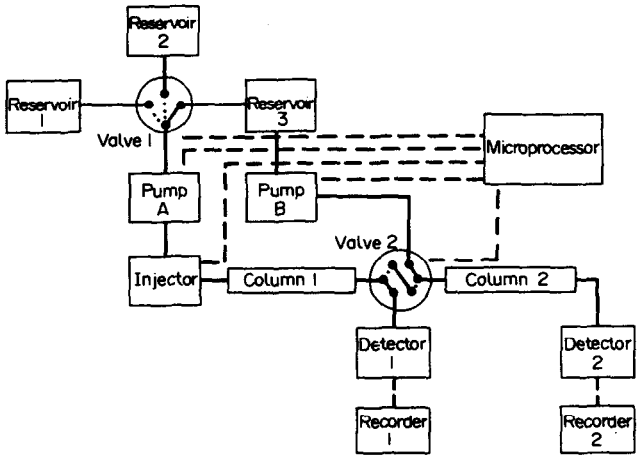
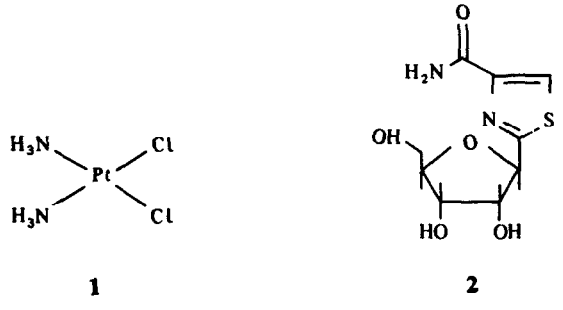


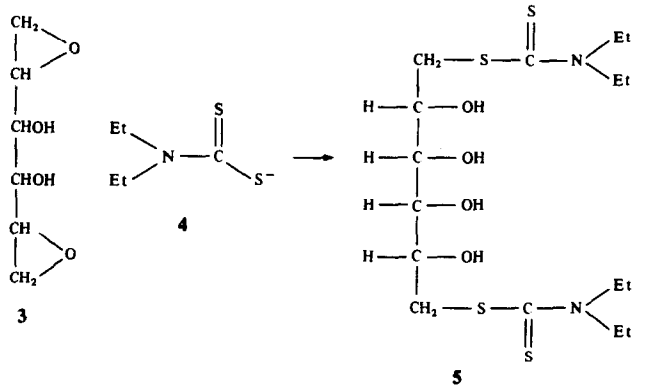
Figure 1
Automated chromatography system for analysis of cisplatin and riboxamide from urine or plasma ultrafiltrate.



1 **2**

diverted to a second column packed with an octadecylsilane (ODS) bonded phase with HTAB adsorbed on its surface. The analytes are subsequently fractionated on the second column and, concurrently with forward flushing of column 1 (silica), directed to the detector. The use of column switching provides rapid, inexpensive sample clean-up that is easily automated; offers powerful separating capacity since it combines column packings of widely different selectivities (on column 1, biological material is strongly retained and drugs **1** and **2** are weakly bound, while on column 2, biological material elutes rapidly and drugs **1** and **2** are strongly retained); minimizes contamination of the analytical column since only the heart-cut containing the compound(s) of interest passes to the reversed-phase column; and shortens analysis times as late-eluting background peaks are not analysed on the second column.

Some drugs cannot be separated from biological fluids by liquid–solid extraction or liquid–liquid extraction: the anti-neoplastic agent, dianhydrogalactitol **3**, is one such compound [3]. Its hydrophilicity prevents efficient extraction into water-immiscible solvents, even from salt-saturated solutions [3]. Furthermore, **3** is unstable, binding irreversibly to red blood cells (even in sample containers) through attack by endogenous nucleophiles at the epoxides, and also undergoing intramolecular rearrangement to the thermodynamically more stable 2,3-epoxy isomer. Analysis is further complicated by the



lack of chromophoric groups providing a detection limit ($\geq$ 10 μg ml^{-1} plasma) that is too high for clinical monitoring of the drug. These difficulties were overcome by derivatizing **3** with diethyldithiocarbamate **4**. Reaction was carried out directly in the blood sample (at the bedside); conversion to the *bis*-dithiocarbamate **5** was quantitative and complete in less than 5 min. Elimination of the epoxides by conversion of **3** to **5** stabilized the analyte from subsequent nucleophilic attack. The derivative chromatographed efficiently on a normal phase (CN) column and absorbs UV light strongly ($a_{m,254\ nm} = 2.8 \times 10^4$), thus providing a sensitive, specific route to clinical monitoring in blood.

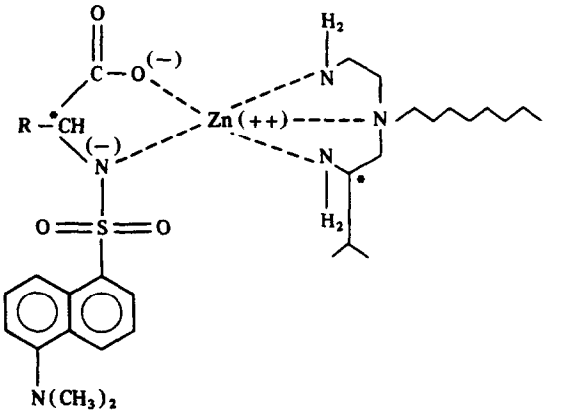
High Efficiency Separation Step

Although specificity can be introduced during sample clean-up as indicated above, it is more commonly introduced in a subsequent chromatographic step. HPLC has emerged as a powerful tool for achieving such selectivity in clinical analysis. For some applications, however, the specificity provided by common HPLC systems is insufficient. For example, in situations where discrimination among enantiomeric mixtures is required, modifications of the stationary or mobile phases have been made to provide chiral recognition. Chiral stationary phases have been prepared and then covalently or ionically bonded to γ-mercapto- [4] or γ-amino-propyl [5] silanized silica microparticles. Such materials have been successfully used to resolve racemates without the need for converting them into diastereomeric mixtures prior to chromatography. Most notable have been the efforts of Pirkle who, using a chiral fluoro-alcohol phase [4], was able to separate enantiomeric sulfoxides, amines, amino acids, alcohols, hydroxy acids, lactones and mercaptans, and on a chiral *N*-(3,5-dinitrobenzoyl)phenyl glycine phase [5] could resolve enantiomeric arylalkylcarbinols.

The use of metal ions for selective separations of optical mixtures in liquid chromatography via ligand exchange chromatography has been established for several years [6]. Optically active chelating exchangers have been bonded to a variety of stationary supports, and after equilibration with an appropriate metal ion, have been successfully employed in the separation of optical isomers [7–9]. D,L-Selectivity is found to be high; however, efficiency and peak symmetry are often poor and may thus be unsuitable for analysis of complex mixtures.

A second approach to resolving optical isomers using metal systems is to add chiral agents to the mobile phase, as demonstrated by Nakazawa and Yoneda [10]. Hare and

Gil-Av [11] have separated some underivatized D,L-amino acids on an ion-exchange column through the direct addition of the L- (or D-) proline-Cu(II) complex to the mobile phase. More recently, Karger [12] has resolved amino acid racemates (as their dansyl derivatives) on reverse phase columns by the addition of the chiral chelate L-2-isopropyl-4-octyl-diethylenetriamine-Zn(II) to the mobile phase. A possible structure of the resulting 'diastereomeric' mixed complex is shown below:



Thus, manipulation of stationary phases and (more often) mobile phases can achieve dramatic specificity advances in drug analysis, not only in optical isomer separation, but in many other separations.

Detection

The specificity and sensitivity required in biochemical analysis can also be achieved by judicious selection of detectors. In some instances, this may involve monitoring the drug itself, while in other cases the drug may require chemical derivatization.

Selective Detection of Drugs Without Derivatization

Cisplatin

Although chromatographic systems have been developed to isolate cisplatin from biological matrices [1, 13, 14], its sensitive, specific detection in HPLC column effluents has required off-line atomic absorption spectroscopy. The chromatographic system resolves the platinum complexes differing in ligand composition and the detector responds only to platinum. This detector has been of great use in studying the complex reactions of cisplatin with amino acids. As shown in Fig. 2, the kinetics of the reaction of cisplatin with metenkephalin (forming a variety of products) could be readily monitored [15].

More recently, Bannister [16] constructed a polarographic detector for cisplatin, in which the reductive current produced by 2-electron transfer of **1** at a hanging mercury drop was monitored. A high degree of specificity was provided by thermostating the detector cell at 60°C, shifting the peak reduction potential of **1** from −125 mV (vs Ag/AgCl at 25°C) to 0.00 V (Fig. 3). At this potential, virtually no compounds normally present in biological fluids (including O_2) are reduced, so a highly specific detector

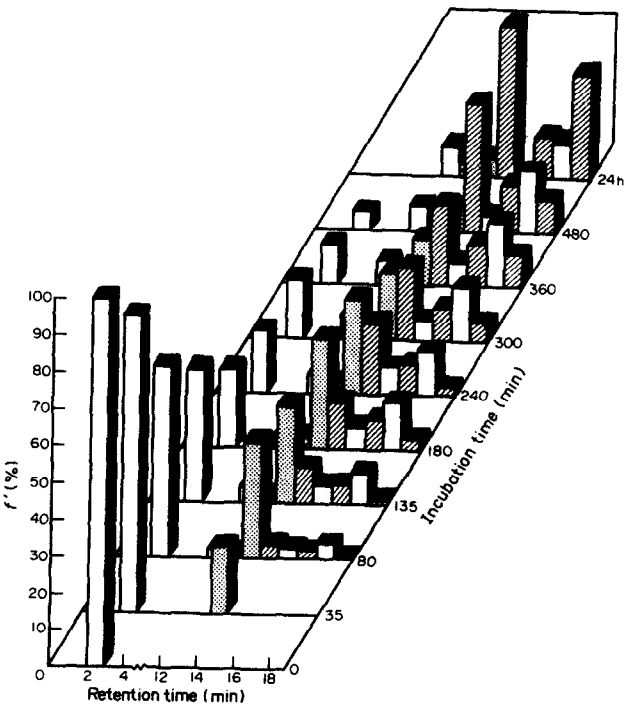


Figure 2
Three-dimensional histogram showing the elution profile of platinum within samples of metenkephalin incubated with cisplatin at 37°C.

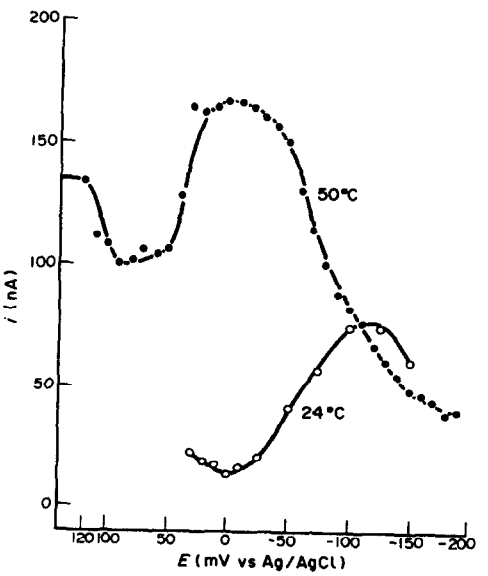


Figure 3
Effect of cell temperature on hydrodynamic polarogram for cisplatin. 1.0s DME, 0.026 cm^2 drop.

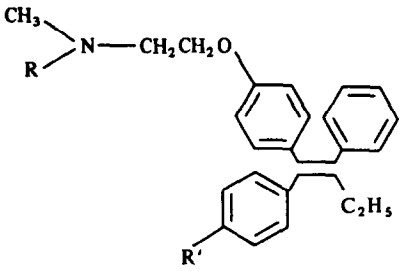
responsive to cisplatin is available for monitoring HPLC separations. By careful manipulation of cell parameters, the noise-limited minimum detectable quantities of cisplatin (S/N = 3) with this system was 70 pg injected. Unfortunately, detector response is highly dependent on the nature of the ligands coordinated to platinum.

Selective Detection of Drugs Requiring Chemical Derivatization

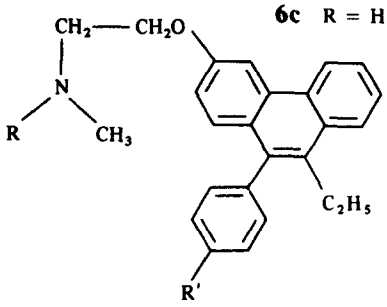
In many instances, drugs lack the physical properties to allow their sensitive and specific detection in HPLC effluents. These properties can be modified and enhanced by chemical modifications of the analyte either prior to or following the chromatographic step. Pre-column derivatization may enhance detectability by increasing response to the detector and by introducing an additional element of specificity through the derivatization reaction (see the example of dianhydrogalactitol discussed above). It has also been utilized to allow clinical monitoring of the anti-estrogen, tamoxifen, and the experimental anti-neoplastic agent, emetine.

Tamoxifen (**6a**) (used in treatment of metastatic breast cancer) and its two major and potentially therapeutically-active metabolites (**6b**, **6c**) have been analysed in whole blood by HPLC with fluorescence detection after photochemically-induced oxidation of the drug species to the corresponding phenanthrene **7** [17, 18].

Following initial extraction from whole blood into ether, the ether was evaporated and the residue reconstituted in HPLC mobile phase. The mixture was then photolysed for *ca.* 10 min and the final mixture (**7a–c**) chromatographed on an RP-18 column. Irradiation conditions were optimized with respect to solvent, distance between light source and reaction vial, and irradiation time. Conversion to phenanthrene was quantitative and formation of **7a–c** followed first-order kinetics ($t_{1/2}$ 2.5–6.5 min). The

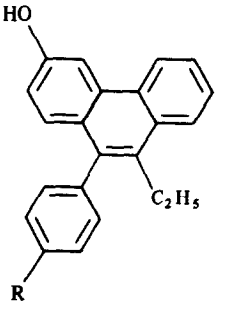


6a R = CH_3 ; R′ = H
6b R = CH_3 ; R′ = OH
6c R = H ; R′ = H



7 (a–c)

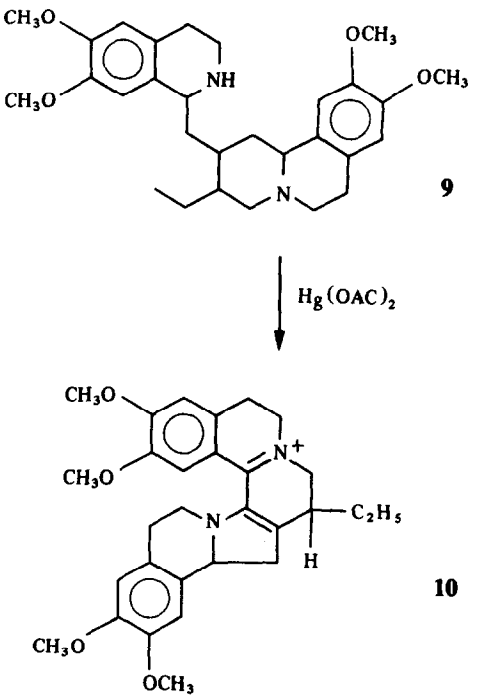
presence of acid in the photolysis mixture inhibited degradation of **7** by protonating the amine nitrogen, thereby reducing its nucleophilicity and preventing intramolecularly-catalysed expulsion of the phenol (**8**) and loss of fluorescence intensity. This method has been more recently extended [19] to allow analysis of tamoxifen and metabolites (**6a–b**) in tissues.



8

Emetine

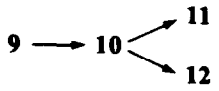
The use of emetine **9** in cancer chemotherapy has been prevented by the severe myopathy and cardiac arrhythmias associated with its use [20]. By administering the drug as a slow i.v. infusion, it appears that these side effects can be avoided. To monitor emetine blood levels attained by this route, a sensitive HPLC-fluorescence assay [21] has been developed based on the mercuric acetate oxidation of emetine (extracted from plasma) to form rubremetine **10**. When heated at reflux temperatures with mercuric



9

10

acetate, emetine degrades (with apparent first-order behaviour ($t_{1/2}$ ~ 15 min) with concomitant formation of a fluorescent product **10**. However, **10** was unstable under these conditions, degrading to two products ($t_{1/2}$ ~ 100 min) **11** and **12**. These formed at a rate similar to the loss of **10** suggesting the following reaction pathway.



The reaction could, however, be controlled by solvent modification. Addition of ethanol to the reaction medium facilitated oxidation of **9** and stabilized **10**. At an optimum level of 70% ethanol, reaction to form **10** was complete at room temperature in 60 min, **10** was stable for several hours, and formation of **11** and **12** was not observed. Levels approaching 1 ng/ml of plasma could be readily quantitated by this technique. Thus, pre-column derivatization may improve both the specificity and sensitivity of HPLC-based analytical procedures in clinical applications.

In some situations, however, pre-column derivatization may *reduce* specificity, and derivatization is more appropriately carried out after the chromatographic step. Such is the case with cisplatin, which is highly reactive *in vivo* forming a variety of platinum-containing degradation products, which may possess a wide spectrum of biological activities [15, 22]. Derivatization of these platinates prior to chromatographic resolution results in their conversion to a common product (i.e. a nonspecific method) [23], so that chemical transformation of individual platinates to readily detectable species must occur 'post-column'. A post-column reactor has been designed [24] (Fig. 4) in which platinates are converted to highly chromophoric products by initial reaction with a solution of sodium dichromate, followed by reaction with sodium bisulfite. Reaction is carried out on-line in braided Teflon coils [25] to minimize band broadening, and the derivative monitored spectrophotometrically at 290 nm. This detector offers sensitivity toward cisplatin (Fig. 5) similar to that of the electrochemical monitor [16], but it also responds strongly to other platinates differing in ligand composition. The method is thus capable of detecting low levels of platinum complexes in biological fluids with a high degree of ligand specificity, thus permitting more detailed studies of the biodisposition of cisplatin and other platinum complexes.

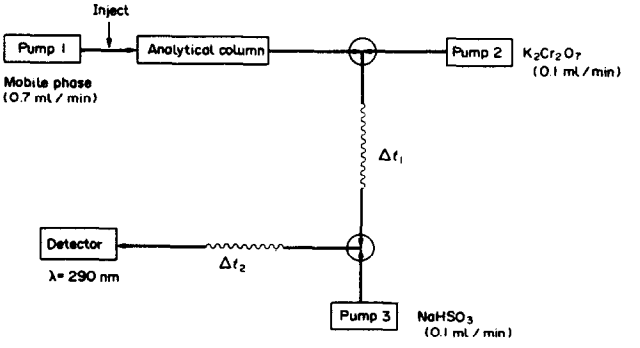


Figure 4
Schematic representation of platinum reaction detector: analyte(s) separated on the analytical column were mixed sequentially with the $K_2Cr_2O_7$ and $NaHSO_3$ reagents and detected at 290 nm after the appropriate reaction time (Δt_1 and Δt_2).

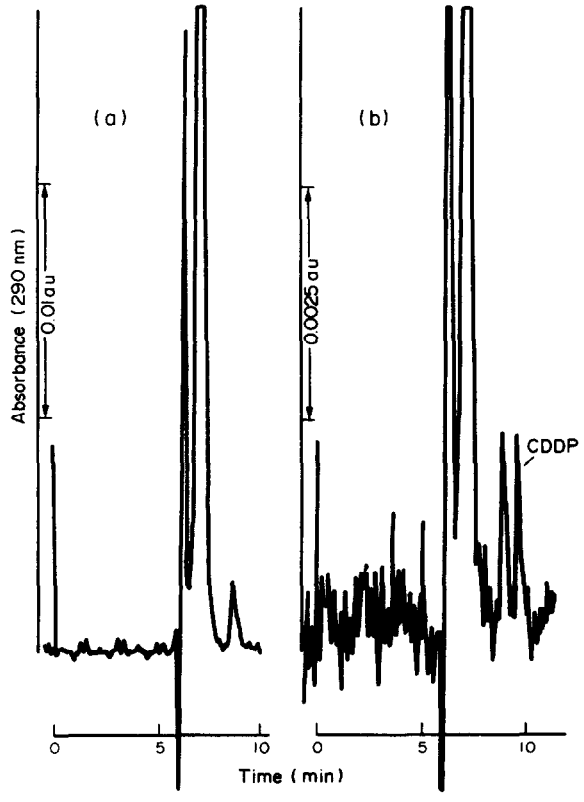


Figure 5
Post-column reactor response to plasma ultrafiltrate blank (a) and plasma ultrafiltrate containing cisplatin (b). Cisplatin peak is the reactor response to 5.1 ng of platinum.

Signal Processing and Data Manipulation

Although a selective HPLC method may be indicated by the presence of a well-resolved, symmetrical peak for the compound of interest, its validity depends on establishing the homogeneity of the peak. Homogeneity, within specified limits, can generally be demonstrated if a critical physical property of the peak does not change with time. In gas chromatography, homogeneity can be determined using a mass spectrometric detector to demonstrate the constancy of the mass spectrum of the peak with time [26]. The homogeneity of HPLC peaks has been demonstrated spectrophotometrically by monitoring the ratio of absorbances at specified wavelengths [27]. Alternatively, peak homogeneity can be evaluated by obtaining higher order (second or fourth derivative) representations of the peak(s) of interest [28, 29]. As the derivative order of the peak increases, so does the achievable enhancement of resolution; the magnitude of this enhancement depends also on band shape, width and height. Nonhomogeneous peaks have been readily deconvoluted using this technique [30].

Conclusions

In summary, we have tried by the presentation of solutions to the clinical analysis of several specific anti-cancer agents, to offer approaches to the analysis of others. The

goals of such endeavours are to impart specificity and sensitivity to analytical methodology, and to ensure reliability and high quality for the data used in pharmacological evaluation of drug therapy.

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