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Liquid chromatography in anticancer drug research with special reference to anthraquinone glycosides*

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Abstract: The need for individualized cytostatic therapy is most apparent in cancer patients with disturbance of the liver or renal function and also in patients undergoing combination drug therapy. Bioanalytical studies can provide a rationale for increasing the therapeutic index by optimization of the dose schedule and by site-specific anticancer drug therapy. Analytical methods based on liquid chromatography are discussed for the anthraquinone glycosides, where the high selectivity of reversed-phase liquid chromatography systems permits ready separation from their 14-hydroxy metabolites. The high sensitivity of photometric and fluorimetric detectors permits quantification in the low ng/ml range. The stabilization of alkylating agents such as melphalan in biological samples by reaction with acetylcysteine is discussed. The melphalan–acetylcysteine derivative, after isolation from the biological matrix by reversed-phase liquid chromatography, can be detected fluorimetrically with high sensitivity and selectivity.

Keywords: *Anthraquinone glycosides; adriamycin; daunorubicin; melphalan; pharmacokinetics; chemotherapy; liquid chromatography.*

Introduction

During the last decade anticancer drugs have become increasingly important in the treatment of neoplastic diseases. Their use has, however, been hampered by the high degree of serious side-effects observed, underlining the need for individualization of anticancer drug therapy, based on the observed plasma and/or urine concentrations of drugs and metabolites. The disposition of anticancer drugs is strongly affected by the status of the patients; such factors as disturbance of the liver or renal function, malnutrition and changes in plasma protein composition are significant. Anatomic abnormalities of the gastrointestinal tract, which are frequent in cancer patients, can modify absorption of orally administered drugs [1].

Most cancer patients treated chemotherapeutically are given various combinations of cytostatics, which makes the pharmacokinetics of the individual drugs even less

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predictable. Moreover, the pharmacokinetics of cytostatics are sometimes time-dependent, i.e. changes are observed with the total dose and number of treatments.

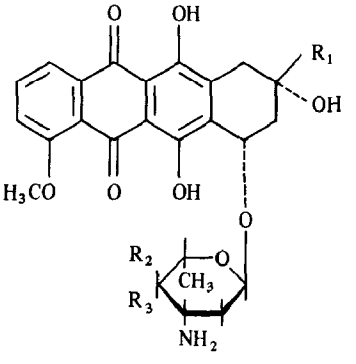
Dose-dependent pharmacokinetics have been reported more frequently for anticancer drugs than for other drugs [2], probably because anticancer drugs are studied over a wide range of doses during early evaluation. Furthermore, in clinical practice a wide range of doses is routinely used. Very high doses of cytostatics are often used in combination with marrow transplantation and/or administration of antidotes [3].

Compared to other drugs a higher number of the cytostatics have to be biotransformed, either intrahepatically or within the tumour itself, prior to exerting biological activity. A multitude of metabolites with various degrees of cytostatic activity are often formed. Clearly, there is a need for highly selective analytical methods for the determination of the anticancer drugs and their metabolites. Analytical methods based on liquid chromatography are generally preferred for most anticancer drugs and their metabolites, due to their chemical and physical characteristics, e.g. low stability, high polarity, low volatility etc.

Anthraquinone glycosides such as adriamycin and daunorubicin are frequently used for solid tumours as well as in leukemia [4]. Their use has been limited by serious side-effects, of which cardiomyopathy is specific for this class of compounds [5]. A number of metabolites of anthraquinone glycosides have been isolated and identified [6], but from the clinical point of view only the 14-hydroxy anthraquinone glycosides derivatives (Fig. 1) are of importance, as they have been reported to exert as high a cytostatic activity as the intact drugs themselves [7, 8]. Successful treatment of leukemia with daunorubicin has been associated with a high degree of formation of daunorubicinol [9].

Figure 1
Structural formulae

Compound	R_1	R_2	R_3
Adriamycin	$-COCH_2OH$	$-H$	$-OH$
Adriamycinol	$-CH(OH)CH_2H$	$-H$	$-OH$
4'-Epi-adriamycin	$-COCH_2OH$	$-OH$	$-H$
4'-Epi-adriamycinol	$-CH(OH)CH_2OH$	$-OH$	$-H$
Daunorubicin	$-COCH_3$	$-H$	$-OH$
Daunorubicinol	$-CH(OH)CH_3$	$-H$	$-OH$



Determination of Anthraquinone Glycosides in Plasma

Sampling

To avoid erronous results when determining anthraquinone glycosides in blood and plasma samples, special precautions have to be taken in the sampling procedure.

The stability of the anthraquinone glycosides is strongly dependent upon pH. Figure 2 indicates that the degradation rate increases with increasing pH. Phosphoric acid (10^{-2}–10^{-1} M) is recommended as a solvent for stock solutions.

Adriamycin and daunorubicin are enzymatically reduced in whole blood to adriamycinol and daunorubicinol, respectively. It is essential to separate the plasma fraction by centrifugation immediately after collection of the blood samples to obtain valid information on the relative amounts of intact drug and reduced metabolite [10]. The

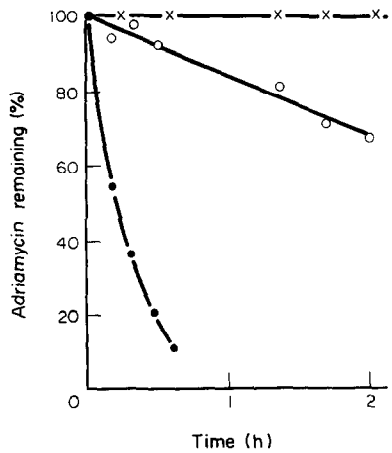


Figure 2
Stability of adriamycin in buffered solution at 37°C. Symbols: × pH 2.0; ○ pH 7.8; ● pH 9.8.

degradation process can also be arrested by treating the blood samples with an ultrasonic cell disruptor.

Studies on the stability of plasma samples containing adriamycin and daunorubicin showed a drastic decrease in the anthraquinone glycoside concentration after repeated freezing and thawing, as well as after storage of spiked plasma samples at −20°C. This effect is most probably not due to degradation of the compounds, but seems to be an effect originating from a change in the plasma matrix. Storage of plasma samples at −80°C does not result in losses of drugs and metabolites.

The plasma half-life time of adriamycin in the alpha-phase is approximately 4 min [11]. Calculations based on these data [11] reveal that high precision in the sampling time is necessary to obtain high accuracy in the calculated pharmacokinetic parameters. The percentage error in reported plasma concentrations of adriamycin with systematic errors in sampling times of ±1 min is illustrated in Fig. 3. The accuracy of reported plasma concentrations varies with sampling time. If the precision in sampling time is ±1 min, the values of reported plasma concentrations fall within ±10% of the correct plasma concentrations ≥30 min post-injection.

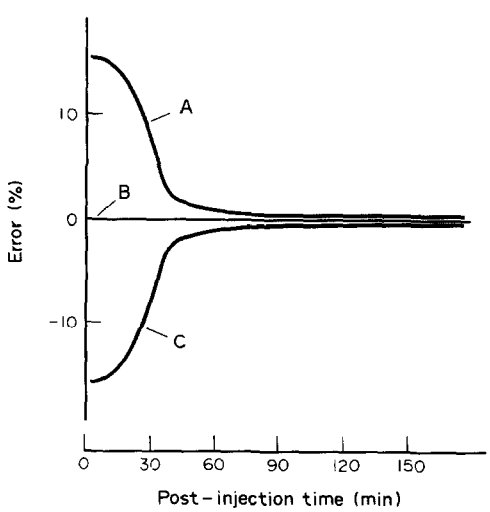


Figure 3
The effect of sampling time on the error in reported plasma concentrations of adriamycin. The error in concentration is shown as a function of time after injection at: A, correct sampling time −1 min; b, correct sampling time; c, correct sampling time +1 min.

The maximum plasma concentration of adriamycin is strongly dependent on the time of administration [12]. A change in the injection time from 1 to 5 min decreases the maximum plasma concentration by a factor of >2. Thus standardization of the administration time is essential to obtain plasma concentration data suitable for pharmacokinetic calculations.

Extraction

In the determination of adriamycin and daunorubicin in biological fluids [13, 14] the anthraquinone glycosides as well as their inactive metabolites, aglycones, are extracted into an organic phase. The active anthraquinone glycosides are re-extracted into an acidic aqueous phase, the aglycones remaining in the organic phase.

The extraction properties of adriamycin and daunorubicin have been extensively studied [15]. The degree of extraction is dependent upon pH and also, due to dimerization and tetramerization processes, upon the concentration of the anthraquinone glycosides in the aqueous phase. Batch extraction was used for the initial extraction of daunorubicin and daunorubicinol, and column extraction for the extraction of adriamycin and adriamycinol, the choice of technique being based on the extraction properties of the compounds.

Reversed-phase liquid chromatography

The separation of anthraquinone glycosides by reversed-phase liquid chromatography has been studied systematically [16]. Acetonitrile is the most suitable organic modifier for the separation of the intact drugs and corresponding reduced metabolites. The retention of the anthraquinone glycosides varies with the concentration of the organic modifier in the aqueous mobile phase, the minimum value of the capacity factor, k', being at a concentration of about 50% v/v (Fig. 4). The selectivity decreases with increasing concentration of the modifiers used, independent of the lipophilic character of the support employed.

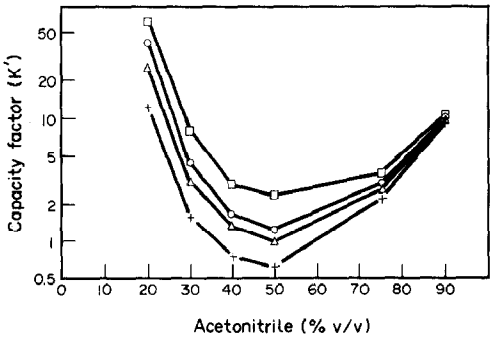


Figure 4
Retention of anthraquinone glycosides as a function of concentration of acetonitrile in the aqueous mobile phase. Symbols: △ adriamycin; + adriamycinol; □ daunorubicin; ○ daunorubicinol. Support: LiChrosorb RP-8; mobile phase: acetonitrile–phosphoric acid (10^{-2} M).

Simultaneous administration of adriamycin and 4'-epi-adriamycin to cancer patients facilitates comparative pharmacokinetic studies of the two compounds. The separation of adriamycin and 4'-epi-adriamycin and their corresponding reduced metabolites is illustrated in Fig. 5. High detection selectivity and sensitivity is obtained by photometric detection at 500 nm, or alternatively by fluorimetric detection (λ_{ex} = 436 nm, λ_f = 550 nm; Schoeffel Instrument model FS 970).

Recoveries of adriamycin, adriamycinol, daunorubicin and daunorubicinol from

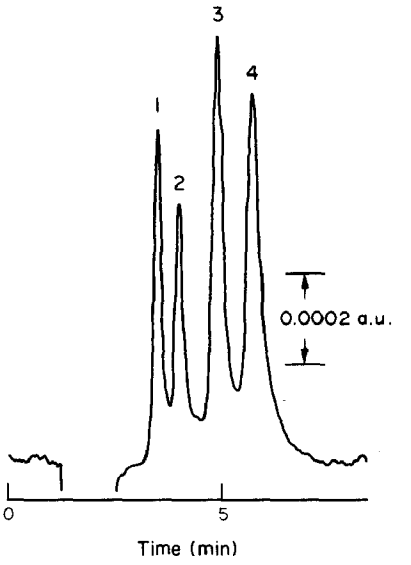


Figure 5
Separation of adriamycin and 4′-epi-adriamycin and their reduced metabolites. Compounds: 1, adriamycinol; 2, 4′-epi-adriamycinol; 3, adriamycin; 4, 4′-epi-adriamycin. Support: LiChrosorb RP-2; mobile phase: Acetonitrile–phosphoric acid (10^{-2} M) (26:74, v/v); photometric detection at 500 nm.

spiked plasma samples were 93, 94, 100 and 100%, respectively, with a precision (RSD) better than ±5% within the range 5–1000 ng/ml, for photometric detection. Calibration graphs for each analyte were linear over this range and regressed through or close to the origin.

Determination of Melphalan in Plasma

Melphalan is an alkylating agent of the nitrogen mustard type used for the treatment of ovarian carcinoma and multiple myeloma. The compound has a low stability in aqueous solutions: at 37°C in phosphate buffer (pH 7) the half-life ($t_{½}$) was 0.75 h, while in plasma it was 2.1 h.

The stability of melphalan can be considerably increased by conversion to the di-acetylcysteine derivative. The derivatization is performed in plasma using 0.1 M acetylcysteine at pH 11, after precipitation of the plasma proteins with trichloroacetic acid. Part of the aqueous phase is injected onto the liquid chromatograph, equipped with a fluorimetric detector (λ_{ex} = 260 nm; λ_f = 360 nm; Shimadzu model RF-530). A chromatogram obtained after the injection of 1.5 ng of derivatized melphalan is shown in Fig. 6. The precision (RSD) at 100 ng/ml is ±5%.

Clinical Applications

Quantification of systemic concentrations of cytostatics and their active metabolites gives little indication of their anti-tumoural activity. One might expect a better correlation between blood or plasma levels and toxicity such as nausea, vomiting, alopecia and myelosuppression [5]. This situation is in contrast to that observed for traditional drug level monitoring, where correlations between blood (or plasma) levels and therapeutic efficacy, as well as the appearance of side effects, are employed for optimization of the dosage and treatment schedules, as for example in the treatment of epilepsy and infectious diseases.

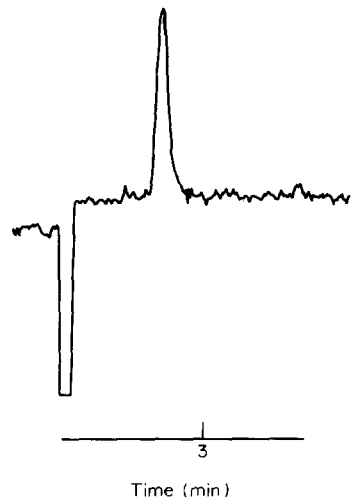


Figure 6
Chromatography of melphalan after derivatization with acetylcysteine. Support: μBondapak-C_{18}; Mobile phase: octane sulphonic acid (10^{-2} M) in citrate buffer (pH 5.7). Fluorimetric detection at λ_{ex} = 260 nm, λ_f = 360 nm.

Loco-regional treatment with adriamycin

Superficial bladder tumours can be successfully treated by intravesical instillation of adriamycin. Standardization of the treatment schedule based on the bladder volume has been introduced [17]. Repeated measurements during and after instillation therapy alone did not reveal any measurable concentrations of adriamycin (detection limit ≤2 ng/ml) [18]. After combined therapy involving intravesical instillation and transurethral resection the plasma concentrations of adriamycin were found to be less than 100 ng/ml [19].

A comparative pharmacokinetic study after intravenous and intrahepatic administration of adriamycin revealed a more than five-fold variation of the area under the plasma concentration–time curve (AUC) and of the maximum plasma concentration (C_p) after dose normalization (AUC/mg/m^2 and C_p/mg/m^2, respectively) [11]. The values of AUC and of C_p were reduced by a factor of 1.5 by intrahepatic administration.

Intrapleural administration of adriamycin is one of the most effective treatments for malignant pleural effusions. Study of the pharmacokinetics in plasma showed that a considerable amount (approximately 80%) of the drug was absorbed from pleura, but also that C_p was more than 15 times lower than after intravenous administration [20]. Only minor side effects were observed after intrapleural administration of adriamycin.

Prolonged adriamycin infusions. Alterations of the adriamycin dose schedule have proved to be one of the most successful methods for increasing the therapeutic index of adriamycin. A study of the relation between C_p, AUC and the infusion time [12] suggests that the optimal time for intravenous administration with respect to side effects is ≥2 h.

Conclusion

It is to be anticipated that the use of pharmacokinetic studies on the anthraquinone glycosides, based on the high-quality bioanalytical data generated by liquid chromatography, together with careful clinical observation of the patient, should lead to improved patient management in this difficult area.

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