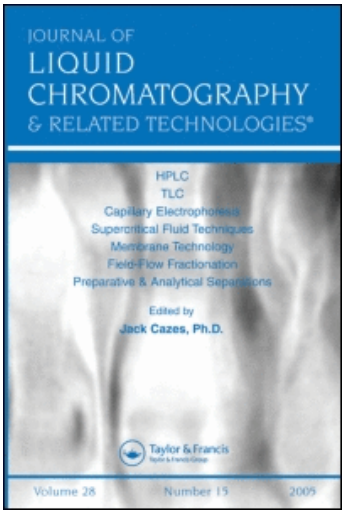

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HPLC Analysis of Adriamycine as N-2,4-Dinitrophenyladriamycine

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To cite this Article Hasson, H. and Warshawsky, A.(1990) 'HPLC Analysis of Adriamycine as N-2,4-Dinitrophenyladriamycine', Journal of Liquid Chromatography & Related Technologies, 13: 8, 1637 — 1649
To link to this Article: DOI: 10.1080/01483919008048981
URL: http://dx.doi.org/10.1080/01483919008048981

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(8), 1637-1649 (1990)

HPLC ANALYSIS OF ADRIAMYCINE AS N-2,4-DINITROPHENYLADRIAMYCINE

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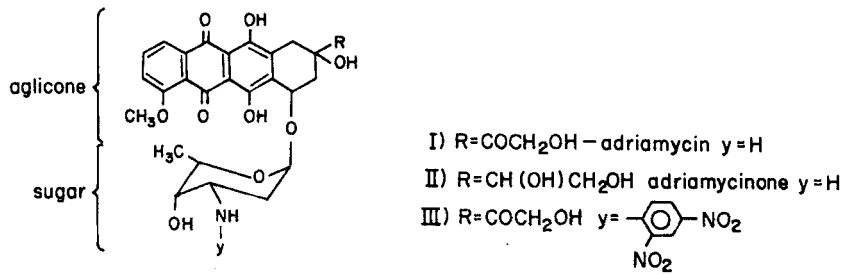
ABSTRACT

Derivatization of adriamycine (I) by reaction with 2,4-dinitrofluorobenzene, followed by HPLC analysis on reversed phase (RP-18 μ-Bondapak) or on normal phase (μ-porasil) and detection at 482 nm, provides a fast method (R_f=4.0 min) for determination of adriamycine [as N-2,4-dinitrophenyladriamycine (III)] in the 1-10 ppm range. Reaction with 2,4-dinitrofluoro[^{14}C]benzene, followed by HPLC separation and detection on a γ counter, extends the detection limit to 0.04 ppm adriamycine.

INTRODUCTION

Adriamycine is an antracycline derivative with antibiotic effects, widely used to produce regression in a variety of neoplastic conditions (1-3). The compound consists of an aglicone called adriamycinone (8-hydroxyacetyl-7,8,9,10-tetrahydro-6,8,10,11-tetrahydrox-1-methoxy-naphthacene-5,12-dione) and a sugar called daunosamine (3-2-amino-2,3,6-trideox-L- lyxo-hexose).

Standard treatment of tumors involves intravenous administration of a large dosage of adriamycine and long term use causes severe side effects. Consequently the idea of replacing the daily administration of drugs with slow release delivery systems which automatically release effective doses from the



Scheme 1: Chemical structure of adriamycine and its major metabolite adriamycinole, and the 2-DNP derivative (III).

protected supply at a controlled rate over a long period seemed very attractive (4). The most common controlled delivery system is a drug encapsulated in a polymeric material. The drug may be released by diffusion through the matrix (5), by erosion of the polymer or by a combination of diffusion and erosion. For the detection of these drugs in vitro and in plasma, a specific direct and sensitive method was needed.

Several analytical methods for adriamycine have been described. Hulhoven and Desager (6) developed a method for the determination of adriamycine, daunomycine (1, R=COCH3) and their metabolites by separation on normal phase column with quaternary solvent mixture as eluent, followed by detection. Barth and Conner (7) separated adriamycine and adriamycinone (I, sugar=hydrogen) from lactose, with visible detection (495 nm) and nanogram range sensitivty, sufficient for metabolic or pharmacological studies.

Eksberg (8) separated adriamycine, daunomycine and their metabolites by reversed phase chromatography, using a 500 nm detector. No data on sensitivity is mentioned.

Quattrone and Ranney (9) extracted adriamycine from plasma, separated it by reversed stationary phase and detected it by a UV detector at 253 nm. Adriamycine was separated from adriamycinone and adriamycinol (II) in about 20 minutes, with a detection limit of 0.2 nmole/ml. Shinozawa et.al.(10) reported separation between adriamycine and its metabolites in serum and tissues in about 15-20 minutes on normal stationary phase, with a fluorescent detector. Other HPLC methods are described in references 11-14.

Some of the methods reported in the literature (7,9,10,12) suffer from long retention times and high flow rates which cause poor resolution and column packing deterioration. Our purpose was not the resolution of metabolites, but rapid and sensitive determination of adriamycine present in low concentrations (ppb to ppm range) such as those anticipated in samples obtained from a polymeric release media.

EXPERIMENTAL

Chemicals

Samples of adriamycine hydrochloride were obtained from Sigma and used without further purification.

The mobile phases were prepared from acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM, Aldrich, HPLC grade) glass filtered through a 0.5 μm filter. Distilled water was passed through a Waters Milli-Q system. Acetic acid and dimethylformamide (DMF) were A.R. grade. 1-Fluoro-2,4-dinitrobenzene (A.R. reagent) from Merck. 1-Fluoro-2,4-dinitro(^{14}C)benzene, from Amersham, specific activity, 65μci/mg. Mobile phases were filtered through 0.5 μm filters (Waters). HPLC columns were 1) RP-18 column μ-bondapak 300x4.6 mm and 2) NP column μ-porasil 300x4.6 mm (Waters). Preparative TLC plates were obtained from Merck (A.R. 5744), 20x20 cm formate with 0.5 mm layer thickness. Scintillation liquid was xylo-fluor from Lumac diluted 1/10 in xylene.

Apparatus

Waters HPLC system with Model 6000A solvent delivery pump, Model U6K injector and Model 480 UV-Vis detector. The output was recorded on an HP model 3390A integrator. The Scintilation counter was Beckman's model LS-1701. ^{1}H NMR spectra were run at 80 MHz on a Varian FT80A instrument. IR spectra were obtained on Matteson CYGNUS 25 FTIR spectrophotometer. MS spectra were taken on Finnigan Model 4020.

Chromatographic Techniques

Reversed phase: Flow rate of 2 ml/min acetonitrile: water 55:45, RP-18, UV detector -253 nm.
Normal phase: Flow rate of 0.8 ml/min 5% MeOH in DCM detector -482 nm.

Assay Procedure

For the reversed phase experiments

2,4-Dinitrofluorobenzene in DMF (5 ml) was added to 25 ml DMF, followed by 3 drops of triethyl amine and 25 ml of a solution of adriamycine·HCl in DMF (0.9 μmole). The reaction was left overnight. 50 μl samples of the reaction mixture were taken for the HPLC analysis.

For the normal phase experiments

To 1 ml solution of 2,4-dinitrofluorobenzene (14 mg, 75 μmole) and triethylamine (15 μl) in DCM (01. ml) and adriamycine·HCl (~0.017 μmole) in methanol was added and the reaction was left for 2 hours. The completion of the reaction was checked by TLC.

The reaction mixture was separated on a TLC plate, using an eluent of 10% MeOH in DCM. The product was stripped and extracted with 1:1 MeOH:DCM, evaporated and redissolved in 1 ml of 10% MeOH in DCM. A 25 μl sample was taken for injection into the HPLC.

Calibration curve (1-10 ppm)

a) In solution: A solution of triethylamine (1 ml) and 2,4-dinitrofluorobenzene (25 mg) in DMF (400 ml) was prepared. To 100 ml of this solution, 1,2,4,6,8 and 10 ml of 100 ppm adriamycine·HCl in DMF was added. After 2 hours stirring, 50 μl sample of the standards were injected into the HPLC and the area of the product peak was monitored.

b) In plasma To 1 ml plasma the following were added successively: 1 drop of 0.1N NaOH, 0.5 ml of 100 ppm adriamycine in water (0.086 μmole) and 4 ml of DCM:Isopropanol 92:8 (extraction method adopted from Ref. 6).

After manual extraction for 1 minute, the tube was centrifuged at 2300 g for 5 minutes. The bottom organic layer was transferred to a Millipore filter in order to remove suspended protein residue and the filter was rinsed with 1 ml of the extraction mixture.

Extraction mixture (2 ml) was added to the residue and after a second extraction, followed by centrifugation, the bottom layer was also filtered. The filtrate was dried in a water bath (30°C) under nitrogen stream. The reaction of the adriamycine with 2,4-dinitrofluorobenzene was carried out in DCM, as described before.

Isolation and Characterization of N-2,4-Dinitrophenyladriamycine (III)

Adriamycine was reacted with 2,4-dinitrofluorobenzene and then evaporated under high vacuum at 45°C. During the neutralization of adriamycine·HCl with NEt_3, the formation of a strongly absorbing violet complex of ADM·NEt_3 was observed The reaction mixture was separated by flash chromatography using 2% methanol in DCM. The unreacted 2,4-dinitrofluorobenzene was recovered in the upfront fraction. The product (III) was found in the fourth fraction.

The IR of III showed the 1583 cm^{-1} NH_2 in plane bending and 815 cm^{-1}, NH_2 out of plane bending, of adriamycine. An absorption at 1509 cm^{-1} (–NH– bending) blurred by the aromatic 1500 cm^{-1} absorption appears along with the absorptions of the 2,4-dinitrophenyl ring: the absorptions at 1607 cm^{-1} (phenyl nucleus), 1509 cm^{-1} and 1360 cm^{-1} (asymmetric and symmetric stretching of the nitro group), 829 cm^{-1} (out of plane bending of ortho aromatic hydrogens), 749 cm^{-1} (C-N-O bending) are also typical for the 2,4-dinitrophenyl group.

In the NMR spectrum of III, the complex ABX type multiplet is visible in the δ = 9-7.5 ppm region, superimposed on the ABC type multiplet of the A ring of the aglicone. The ratio of total aromatic protons of both rings, in comparison to the OCH_3 singlet, is 2:1 as expected. Mass spectrometrical analysis (chemical

ionization or electron impact) was unable to produce a molecular peak at m/e = 709. Cleavage of the ether group linkage between the 2,4-dinitrophenyldaunosamine and adriamycinone in two fashions was observed – leading to fragment A of m/e = 397 and thereon to fragment B of m/e = 379; and through cleavage to fragment C of m/e = 296.

Preparation of Radioactive 2,4-Dinitrophenyl Derivative of Adriamycine (III)

The reagent solution contained 15 mg triethylamine, 14.5 mg 2,4-dinitrofluorobenzene (78 μmole) and 50 μci 2,4-dinitrofluoro[^{14}C]benzene (4.1 μmole) in 2 ml DCM. 20 μl of this solution gave a reading of 228018 cpm in the scintilator. To 0.2 ml (0.038 μmole) and 0.4 ml (0.076 μmole) of adriamycine a solution of 5.5 mg in 50 ml MeOH (20 and 40 ppm adriamycine) 600 μl (25 μmole) reagent were added. After two hours, the reaction was completed (TLC). In order to avoid loading the column with radioactive material, the reaction mixture was first separated on TLC preparative plate (eluent 5% MeOH in DCM) and the orange product was stripped from the plate and extracted with 1:1 DCM:MeOH. The extracted solution was evaporated and dissolved in 500 μl eluent. 100 μl was injected into the HPLC. The 4 minute peak areas, as detected by UV, were 17075 (arbitrary area units) for the 20 ppm sample and 38721 for the 40 ppm sample. The effluent coming out of the HPLC was collected in 1 min (0.8 ml) fractions into scintilation vials, evaporated in a hood and read in a scintilator for 1 min, giving 2070 cpm for the 20 ppm sample and 4307 cpm for the 40 ppm sample as expected.

RESULTS AND DISCUSSION

The reaction between amines and 2,4-dinitrofluorobenzene (2,4 DNFB) proceeds rapidly at "normal" concentrations as described by Sanger (15). Whether this reaction can proceed with adriamycine (ADM) under very low concentrations of ADM must be determined. The rate of reaction was studied in 1-100 ppm range of adriamycine and 44 to 187 molar excess of 2,4-DNFB in DMF, CH_2Cl_2 (DCM) and $CHCl_3$. Since ADM is readily soluble in water and in DMF and practically

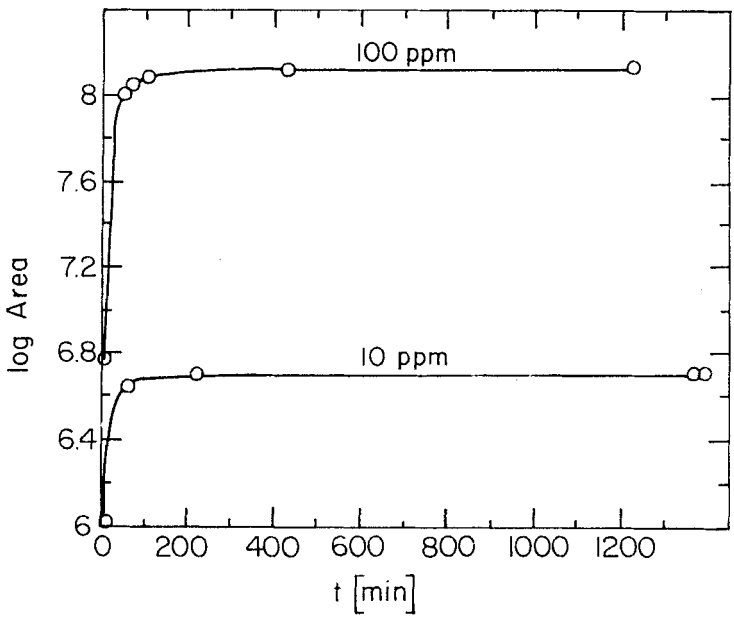


Figure 1: Plot of the rate of formation of III at 10-100 ppm ADM. Conditions: RP-HPLC of 5 ppm adriamycine, 2.8 ml/min, 26% acetonitrile in water containing 20 mM acetic acid, inj. 50 µl, 253 nm, max 0.0-0.014.

insoluble in all other solvents, the method chosen was to dissolve adriamycine in methanol and add the 2,4-DNFB in another solvent.

The rate of reaction between adriamycine and 2,4-DNFB in DMF is shown in Fig. 1 for 10 and 100 ppm ADM. The reaction times needed are 100 minutes or more. This is correct also for 1 ppm ADM (not shown in the figure). First, a reversed phase column method for the determination of III was developed. By this procedure, the early peaks at <6 min were identified as 2,4-dinitrofluorobenzene and its hydrolysis product, 2,4-dinitrophenol in addition to dimethylformamide and triethylamine. The Calibration curve for 2,4-dinitrophenyladriamycine showed good linear response (Fig. 2).

Since adriamycine is very polar and is strongly retained on a normal phase column, most of the separations of adriamycine and its metabolites found in the literature were run on a reversed phase column. Since N-2,4-dinitrophenyladria-

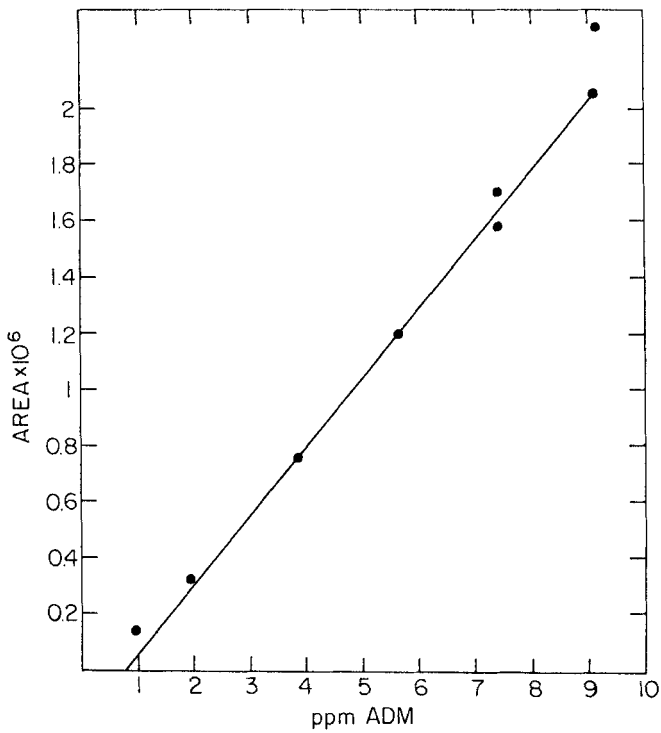


Figure 2: Calibration curve for 1-10 ppm of adriamycine as compound III.

mycine (III) is much more hydrophobic, we expected better separation properties on a normal phase column.

The derivatization of adriamycine was carried out in DCM, and III was separated on a normal phase column by elution of 5% MeOH in DCM at a rate of 0.8 ml/min and detected at 483 nm. The chromatograms are shown in Fig. 3. The area under the 4.0 min peak is constant and does not change with the variations in adriamycine concentration. Hence this compound is the 2,4-dinitrofluorobenzene or its hydrolysis product. The area under the peak at 6.55 min is linearily proportional to the concentration of III.

The detection limit (1 ppm) of the HPLC method may be further expanded if radiolabelled 2,4-dinitro-[^{14}C]benzene is added to the reaction mixture. To demonstrate this point two samples containing 20 and 40 ppm adriamycine were

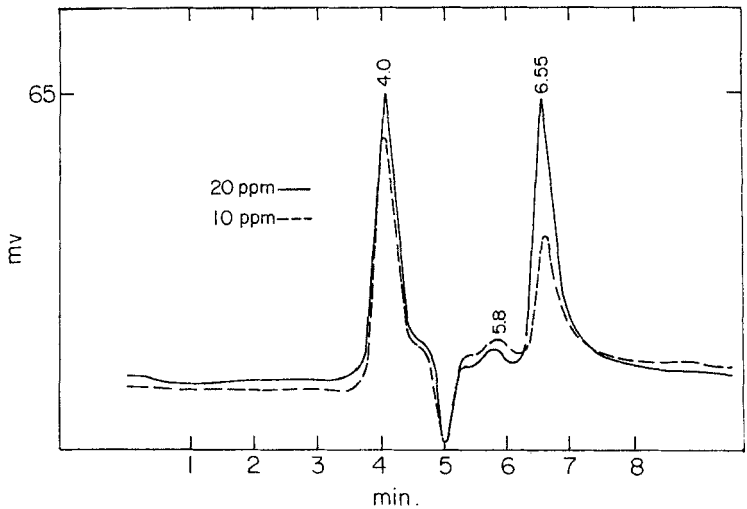


Figure 3: Chromatogram of 2,4-dinitrophenyl adriamycine on normal phase.N.P. 0.8 ml/min, 5% methanol in dichloromethane, detection 483 nm.

reacted with 657 molar excess of "2,4-dinitrofluorobenzene carrier" containing 5% of radiolabelled 2,4-dinitrofluorobenzene. The peak areas as detected by the UV-VIS detector were 17075 for the 20 ppm and 38721 for the 40 ppm sample. The effluent coming out of the HPLC was collected as one minute (0.8 ml) fractions in scintilation vials. The readings were 2079 cpm and 4307 cpm for the 20 and 40 ppm samples respectively.

Fig 4 shows the HPLC chromatogram of a reaction mixture of 40 ppm adriamycine as detected by U.V. detector. Superimposed in the dashed box are the scintilation counter readings. Superimposed in the spotted box are the scintilation counter readings of a reference sample containing no adriamycine.

To further reduce cross contamination between radiolabelled reagent and product, the reaction mixture was separated first from the large excess of unreacted 2,4-dinitrofluorobenzene (and its hydrolysis products) by a preparative TLC on silica (see experimental). This also helps in reducing the load on the column and ensures longer usage of the column.

In order to increase the working range of the radiolabelled detection, it was necessary to carry out experiments with varying ratios (x44, x88, x177) excess

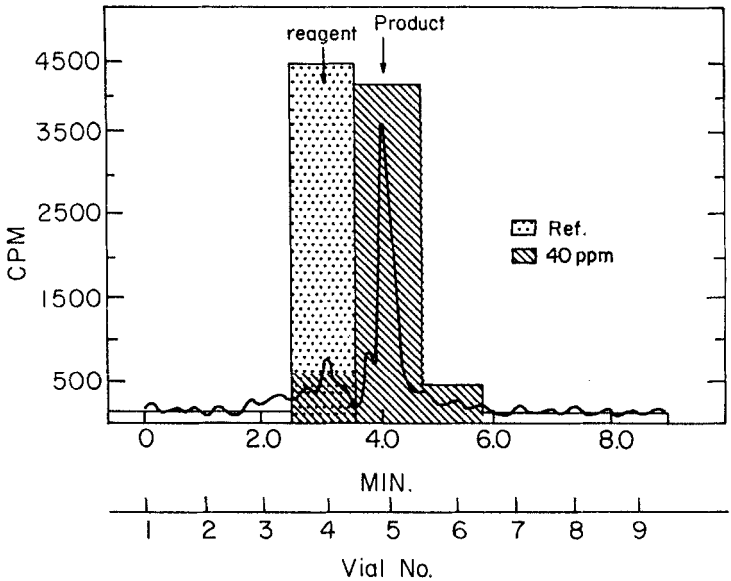


Figure 4: Chromatogram of adriamycine 2,4-dinitrophenyl (III) superimposed on the scintillation counter, results (in boxes). The chromatographic conditions: N.P. 1 ml/min, 5% methanol in DCM, 483 nm.

of 2,4-dinitrofluorobenzene and adriamycine (10 ppm) in DCM at 34°C. Compound (III) was formed with x50 excess of 2,4-dinitrofluorobenzene after 7 hours. Increasing the reaction temperature to 64°C ($CHCl_3$) showed no improvement. Assuming readings of approximately 200 cpm (with background of 6-10 cpm) and samples of 100 µl for injection into the HPLC, this represents a detection limit of about 0.04 ppm of 2,4-dinitrophenyladriamycine (III).

Finally, the method was tested on plasma samples: 10 and 50 ppm samples of adriamycine (0.086 and 0.43 µmole) extracted from 1 ml plasma with DCM:isopropanol 92:8 mixture according to the procedure reported in ref 6 (90±3% extraction) were reacted with 2,4-dinitrofluorobenzene (32 µmole) in DCM and injected into the HPLC. A chromatogram of a sample of 50 ppm adriamycine, analyzed on reversed phase are shown in Fig. 5.

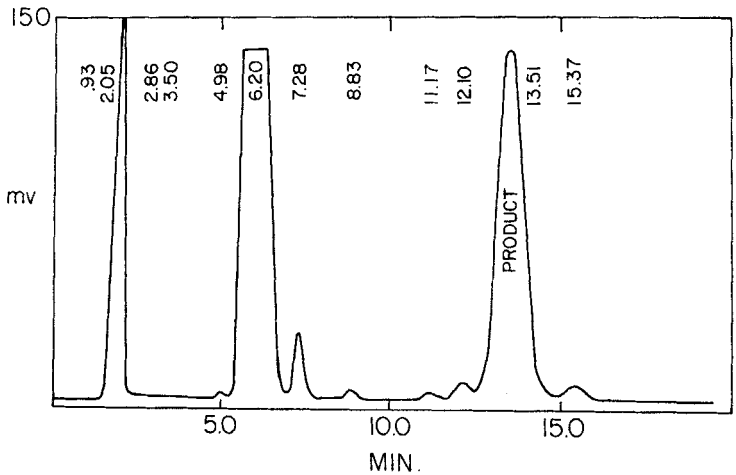


Figure 5: Chromatogram of plasma samples (a) reference (no adriamycine), (b) 50 ppm adriamycine (as compound III). RP, 1 ml/min, 45:55, acetonitrile:water, detection 483 nm.

CONCLUSIONS

In conclusion, the 2,4-dinitrophenylation of adriamycine provides a rapid (R_f=4 min on normal phase), low pressure method for determining the adriamycine in the 1-50 ppm range. Using radiolabeled 2,4-DNFB extends the sensitivity to 0.04 ppm ADM. A further increase in sensitivity is limited due to the need for a large excess of 2,4-DNFB. The method is extended to analysis of ADM extracted from plasma.

ACKNOWLEDGEMENT

Taken in part from the M.Sc. thesis of H. Hasson, The Feinberg Graduate School, The Weizmann Institute of Science, Rehovot, Israel, 1989.

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