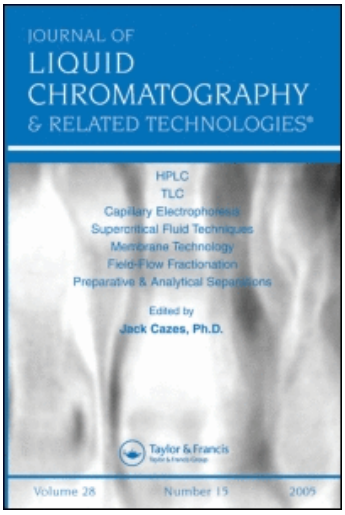

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of MTDQ in Human Plasma by High Performance Liquid Chromatography

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To cite this Article Fekete, J. , Pungor, E. , Nagy, G. , Kocsi, E. , Csokán, P. and Ziegler, F.(1988) 'Determination of MTDQ in Human Plasma by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 11: 9, 2019 — 2038
To link to this Article: DOI: 10.1080/01483918808069038
URL: http://dx.doi.org/10.1080/01483918808069038

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(9&10), 2019-2038 (1988)

DETERMINATION OF MTDQ IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and selective normal phase chromatographic determination method is described for the determination of MTDQ / Sensorad^{R}/ in human plasma. The drug was extracted into n-hexane-isoamylalcohol mixture (95,5/o,5 v/v) using a Partisil 5 µm chromatographic column, with n-hexane-methylenechloride-dioxane mixture (60/40/0,5 v/v) as eluent. Separation and quantification of MTDQ have been achieved within 15 min. The detection limit of the compound was 1 ng at 254 nm detection wavelength. The recovery was 90,4% and the coefficient of variation was found to be less than 4,5%

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x Partly presented at the 5th American-East European Chromatographic Symposium, held in Szeged /Hungary/ 10-14th September, 1984, and later extended in detail.

in the concentration range of 0,01-10 µg/ml MTDQ in plasma. No drug, drug metabolite or endogenous substance interference was observed. Data on steady-state plasma concentration during long-term admonistration of the drug are presented. The effective concentration of MTDQ in plasma proved to be 1-7 µg/ml.
The reproducibility and precision of normal and reversed phase chromatography of the drug were compared.

INTRODUCTION

MTDQ is a novel 1,2-dihydroquinoline derivative /6,6'-methylene-bis-2,2,4-trimethyl-1,2-dihydroquinoline, trade name Sensorad^{R} /being used in tumor therapy /Fig.1.//1-7/. MTDQ undergoes photooxidation very easily /FIG.2.//8/. Recently Belanyi and coworkers,using ESR spectroscopy, have shown another pathway of oxidation. MTDQ has been found to form also stable N-oxide radicals /4/.

Until now, no reliable, selective and sensitive method of MTDQ determination in blood, plasma or serum has been published.
UV-VIS spectroscopy and thin-layer chromatography /9/ give only semiquantitative or even erroneous results. The main cause of error is the interference by other components in case of spectrophotometry /for it is not a selective method / and photooxidation in solution. To avoid this, polar extraction solvents must not be used. Diethylether, being a frequently used extraction solvent, always contains peroxide as stabilizer. It can oxidize MTDQ as shown below.
Polar solvents can dissolve a considerable amount of oxygen which readily oxidizes MTDQ. On the other hand solubility of oxygen in an apolar solvent, as n-hexane

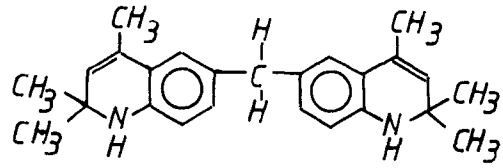

Fig.1. Structure of MTDQ /Sensorad^{R}/

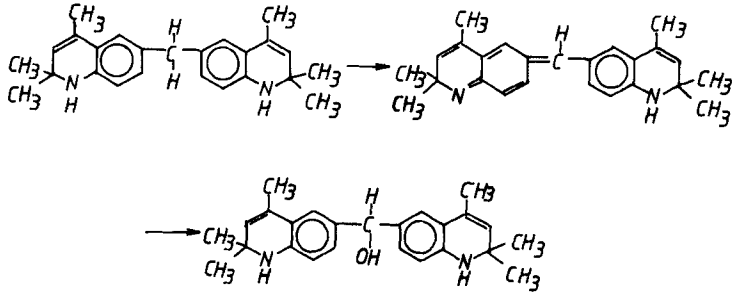

Fig.2. Photooxidation of MTDQ in Solution

is negligible. N-hexane can be easily purified by distillation and by running through a silica gel column.

Extraction of MTDQ into *n*-hexane-isoamylalcohol and normal phase chromatographic separation are described below. The precision and reproducibility of the extraction are discussed. The sensitivity, accuracy, linearity of normal and reversed phase systems are evaluated.

Both methods were applied for monitoring plasma MTDQ concentrations during long term therapy, and values obtained are compared.

EXPERIMENTAL

Reagents and Materials

Pure reference samples of MTDQ were supplied by Humán-Works /Budapest,Hungary/. Organic solvents used were n-hexane, methylenechloride, diethylether, dioxane and methanol. Water was double distilled.All solvents were analytical grade /Merck,Darmstadt,FRG/ except methanol which was chromatographic grade. Distilled n-hexane was used for extraction. It was further purified by passing through a 20x2 cm glass column packed with activated silica gel /Kieselgel 60 Merck,Darmstadt/. The activation temperature was 210°C. The silica gel was fractionated by sieving, and the 100-200 µm fraction has been used.

Instrumentation

During the experiments two chromatographic systems were applied:

I.Normal phase chromatography - The system comprised of a Du Pont 830 high pressure pump /Du Pont, Wilmington, DE.USA/, a Rheodyne 7125 loop injector /Rheodyne,Berkeley CA. USA/, a constant wavelength UV detector /Du Pont/ and a variable wavelength UV detector /BT 3030, Biotronik, Maintal, FRG/. For separation, a stainless-steel column /250x4,6 mm/ packed with Partisil 5 /partical size 5 µm /Whatman, Clifton, USA/ has been used.

II.The reversed phase system was composed of a Waters 6000A pump /Waters, Milford,USA/,a Rheodyne 7125 loop injector, a variable wavelength UV detector /BT 3030, Biotronik,Maintal, FRG/, anda stainless-steel column packed with Spherisorb ODS /partical size 5µm/ /Chrompack, Middelburg, the Netherlands/.

Preparation of standards

All standard solutions were stored in light-resistant /amber glass/ containers. Diluted solutions were always prepared freshly, and discarded after injections.
For normal phase chromatography, approximately 5 mg of MTDQ was weighed into a 25 ml amber glass container and dissolved in n-hexane with ultrasonication.
For quantitative analysis of MTDQ in plasma, standard solutions were prepared by dilution of a stock solution stored in refrigerator. Working concentrations were between 0,1-10 µg/ml.For reversed phase chromatography, approxymately 5 mg of MTDQ was dissolved in oxygen-free methanol in an amber glass container. It was diluted to give a concentration range of 1,1-10 µg/ml. After usage both stock and diluted standard solution were discarded.

Preparation of eluents and extraction solvent

For normal phase chromatography dioxane and methylenechloride were distilled and purified before use by passing through a column of activated silica gel. Before preparing the eluent, argon gas bubbled through all solvents.

For reversed phase chromatography methanol and water were degassed with the aid of a vacuum pump, ultrasonication and with a stream of argon gas /30ml/min/ . A line filter from a Waters 6000A pump-inlet served as an efficient aspirator for the gas.

N-hexane and diethylether used for extraction were distilled and purified by activated silica gel /see above/.

Chromatographic conditions

The composition of normal phase eluent was: n-hexane-methylenechloride-dioxane (60/40/0,5v/v). A flow rate of 1 ml/min was used at ambient temperature /approx. 1200 psi pressure drop/. Detectionwavelengths were 254 and 240 nm, respectively. In case of reversed phase chromatography the eluent was methanol-water (85/15 v/v). A flow rate of 1ml/min was used at ambient temperature.Detection wavelengths were 240 and 254 nm, resp.

Blood sampling and handling

Blood samples were collected by dr.J.Farkas in the National Institute of Oncology /Budapest,Hung./ from hospitalized patients. Clot-formation was inhibited by heparine. The blood was centrifuged at 1200 relative centrifugal force /C.R.F./ and the plasma was transferred to 5 ml glass centrifuge tube and extracted with n-hexane n-hexane-isoamylalcohol (95,5/0,5 v/v) mixture or diethylether. The tube was shaken by a Vartex mixer /Kutesz type, Hungary/ for a minute and centrifuged. The upper layer was removed by means of disposable Pasteur pipette, and the plasma was extracted again with 2,5 ml of organic solvent. The combined organic phase was transferred to a light-resistant tube and evaporated to a volume of 0,5 ml under stream of nitrogen at ambient temperature. 100 µl extract was injected directly onto the Partisil 5 column.

In case of reversed phase chromatography, combined organic phase was evaporated to dryness. The residue was dissolved in eluent and injected onto the Spherisorb ODS column.

RESULTS AND DISCUSSION

As illustrated by Fig.3. precautions /amber glass containers, argon gas flushing/ and the use of an apolar solvent for dissolution were needed to avoid or to minimize photooxidation of MTDQ at low concentrations. Photooxidation in polar solvents /methanol, ether/ is very pronounced and the degree of degradation depends on the MTDQ concentration.

Representative chromatograms of MTDQ test compound are shown in Fig.4. When using reversed phase system, photooxidation products are always present in methanol solutions. The ratio of MTDQ and by-products varies randomly at µg level. One possible reason for this is that there is no way to check the residual dissolved oxygen in polar solvents - even after thorough degassing - and oxygen can degrade MTDQ in the presence of light. The oxidation mechanism - no subject of this study - is complicated, several simultaneous reactions may occur. As shown in Fig.5. , more than five degradation products are formed in methanol solution. A more detailed study of MTDQ photooxidation is under way.

To avoid erroneous results, solvents with poor oxygen dissolution power are recommended. The solubility of oxygen in n-hexane is negligible, therefore the risk of getting erroneous results is greatly reduced.

At 254 nm the detection limit was 1 ng, corresponding to 10 ng/ml MTDQ concentration in plasma. Using a 100 µl loop only a slight loss of column efficiency was observed with time.

The recovery of MTDQ was 90,4 $\pm$ 4,3% /n=9/ (see TableI.)

In Fig.6. a blank plasma chromatogram, in Fig.7. a patient-plasma chromatogram are shown respectively.

In Table II. the MTDQ concentration in blood plasma is recorded during long term administration.

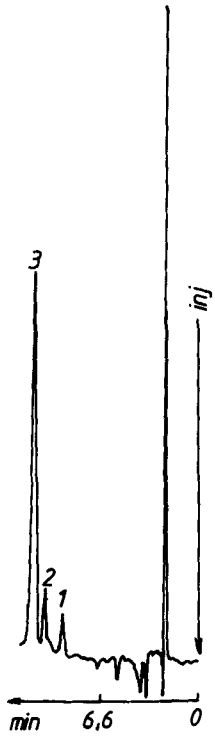

Fig.3. Normal phase determination of MTDQ
Column: Partisil 5 µm /250x4,6 mm/
Eluent: 60/40/0,5 v/v n-hexane/methylene-chloride/dioxane
Flow rate: 1 ml/min
Detection wavelength: 254nm
Compound: 1 and 2 photooxidation products of MTDQ
3 MTDQ
Amount injected: 60 ng

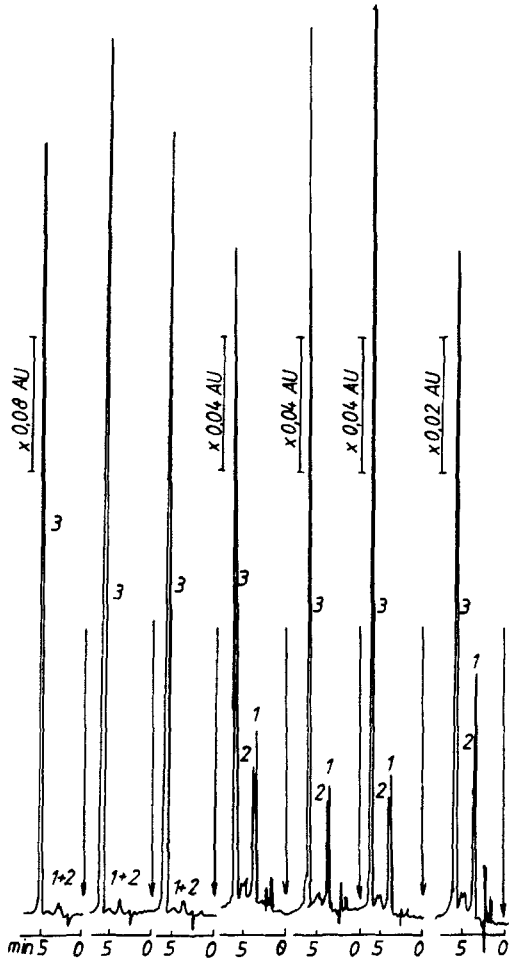

Fig.4. Reversed phase determination of MTDQ
Column: Spherisorb ODS 5 µm /250x4,6 mm/
Eluent: 85/15 v/v methanol/water
Flow rate: 1 ml/min
Detection wavelength: 240 nm
Compounds: 1 and 2 photooxidation products of MTDQ
3. MTDQ
x unidentified photooxidation by--products
Amount injected: A. 0,5 µg B. 1,4 µg
C. 1,3 µg D. 1,0µg E.F. 5,0 µg

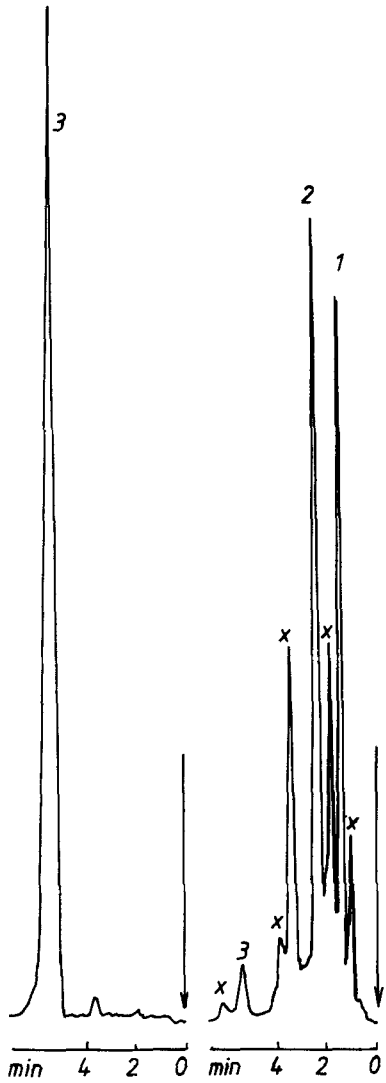

Fig.5. Photooxidation of MTDQ in methanol-water mixture.
Detector: wall-jet amperometric detector
Detection potential: +0,8 V (vs Ag/AgCl)
Eluent: 85/15 v/v methanol/buffer (0,01M KH_2PO_4)
Column: Spherisorb ODS 5 µm
A. Injected directly after dissolution
B. Injected 5 minutes after dissolution

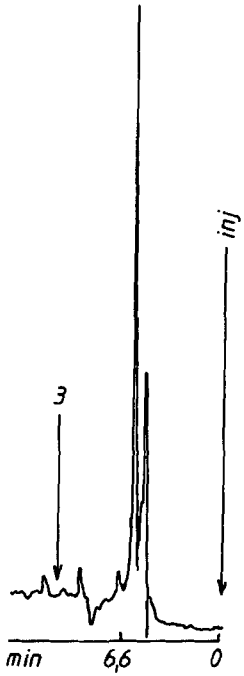

Fig.6. Blank plasma. For conditions see Fig.3.

The average plasma concentration of the drug is given in Fig.8.

Results of RP HPLC experiments are summarized in Table III. The coefficient of variation is higher in this case, compared to normal phase system. As shown in Figs.9.and 10., the risk of interference by endogenous compounds is also higher in methanol/water eluent and - as stated above - photooxidation may occur and the results are not reliable and reproducible.

In our study using diethylether for extraction the recovery of MTDQ was 55 $\pm$ 10% /n=5/. The reduced

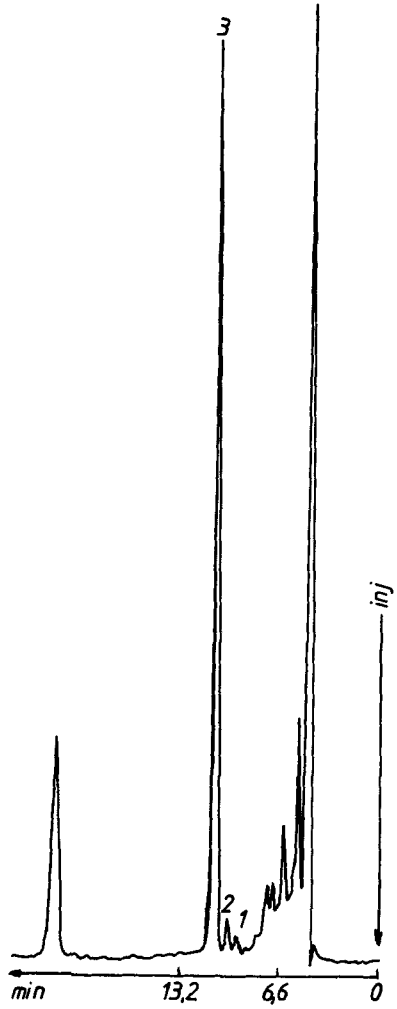

Fig.7. Determination of MTDQ in plasma. MTDQ level of plasma after 21 days of administration is 2733 ng/ml. For conditions see Fig.3.

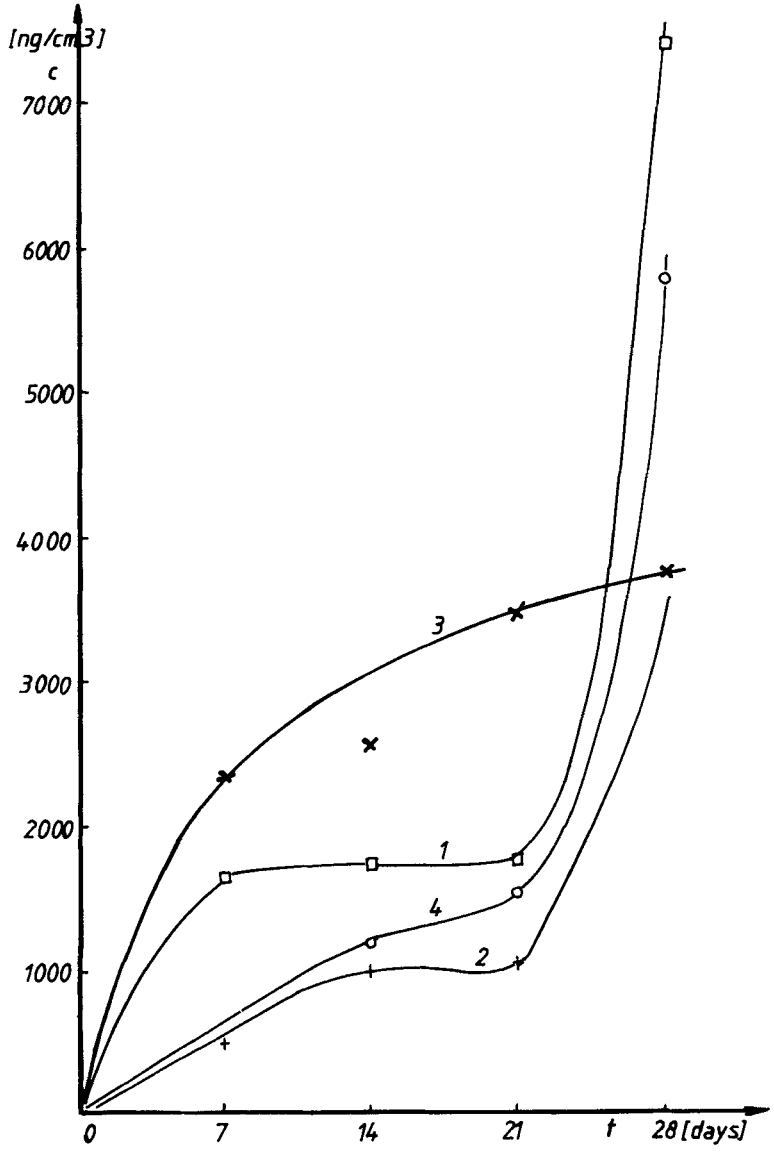

Fig.8. Plasma concentration during the MTDQ administration /4 patients/

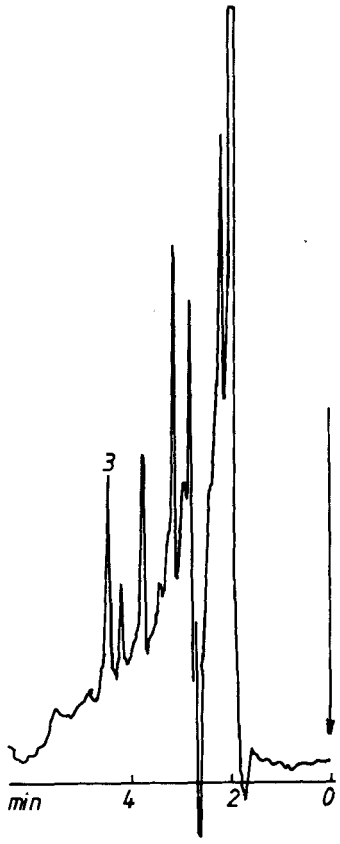

Fig.9. Reversed phase determination of MTDQ in plasma
Plasma concentration found using RP HPLC is 1650 ng/ml, true value is 1743 ng/ml determined by NP HPLC.
Column: Spherisorb ODS 5 µm
Eluent: 90/10 v/v methanol/water
Detction wavelength:240 nm
Flow rate: 1 ml/min

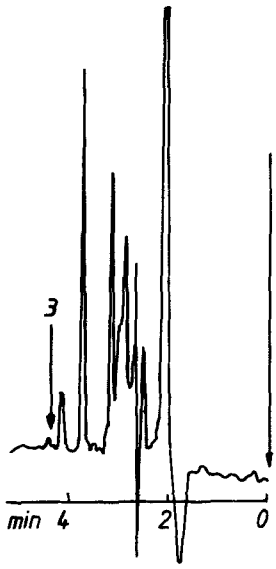

Fig.10.Plasma blank using RP HPLC.
For conditions see Fig.9.

recovery of the compound can be partly attributed to the peroxide present in diethylether which causes oxidation of MTDQ.

CONCLUSIONS

For monitoring the MTDQ level of plasma, normal phase chromatography is recommended. An apolar extraction solvent - n-hexane - has been used for extraction. By its use reliable and reproducible results are obtained and the coefficient of variation was also acceptable.

Reversed phase chromatographic experiments may give erroneous results because of the photooxidation of MTDQ in hydro-organic mixture used to dis-

Table I.

Recovery of MTDQ

N° of sample	Recovery /%/
1	92,7 ± 2,7
2	92,3 ± 6,5
3	94,4 ± 2,6
4	90,0 ± 4,6
5	90,0 ± 5,7
6	84,0 ± 6,0
7	92,5 ± 0,2
8	90,3 ± 4,4
9	87,0 ± 6,0
Average:	90,4 ± 4,3

Table II.

Blood plasma concentration of MTDQ during long term administration (ng/ml)

Results of NP HPLC

term of adm. days / N° of serum	7	14	21	28
1	1667±72	1743 ±74	1766±76	7333±315
2	500±21	967±42	1000±43	3450±148
3	2333±100	2500±108	3400±146	3733±161
4	667±29	1200±52	1500±65	5800±249

Table III.

Blood plasma concentration of MTDQ

obtained by RP HPLC

(ng/ml)

term of adm. days / N° serum	7	14	21	28
1	1625±300	1650±250	1705±300	7055±350
2	435±165	1025±235	1062±338	3362±252
3	2325±342	2450±365	3322±352	3625±342
4	-	1059±243	1523±378	3658±438

solve and elute the compound. The use of diethylether as extractant is very critical. The peroxide present in ether oxidizes MTDQ, which is manifested in the poor precision and reproducibility and low recovery. However, it is rather difficult to obtain peroxide--free ether.

Normal phase separation of MTDQ provides sensitive and precise results when monitoring the drug concentration in plasma or serum. The detection limit is 10 ng/ml. Steady-state plasma levels /1-7 µg/ml/ found exceed 100 times the detection limit of the method. Steady-state plasma levels might be affected by age, sex, genetics, actual condition and by several other factors of patients. Investigation of these needs further experimental work.

ACKNOWLEDGEMENTS

The authors wish to thank Miss K.Farkas for her assistance and preparing the manuscript, dr.J.Farkas for donating blood samples. This work was supported by Innovagent /Budapest,1369 P.O.Box 360,Hungary/.

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