7,7,8,8-Tetracyanoquinodimethane as a new derivatization reagent for high-performance liquid chromatography and thin-layer chromatography: rapid screening of plasma for some antidepressants

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Abstract

Using 7,7,8,8-tetracyanoquinodimethane (TCNQ) as a new derivatization reagent for HPLC and TLC, novel methods are described to detect secondary amine-bearing antidepressants (paroxetine, desipramine, fluoxetine, nortriptyline, maprotiline). The HPLC method is sensitive enough to detect these drugs in plasma at therapeutic levels whereas the latter has potential to detect them in overdose or forensic cases. The methods are based on purple chromogens formed by the displacement reaction of the drugs with TCNQ. The resulting chromogens are directly separated by either reversed-phase HPLC on a C_{18} column or TLC on silicagel plates. For HPLC, acetonitrile–water (60:40) was used as mobile phase, with detection at 567 nm and separation in 40 min. For TLC, three developing solvent systems were used. By HPLC, 36 ng ml^{-1} spiked plasma concentration of the drugs gave easily detectable signals whereas by TLC, detection limits varied mostly between 240 and 480 ng ml^{-1}. The HPLC method was applied to real plasma samples. The methods described are simple and very selective; some metabolites of these antidepressants and a vast number of drugs do not interfere with detection. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Up to date, TCNQ as an analytical reagent has only been used for the spectrophotometric determination of some drugs such as alkaloids [1], penicillins [2], antihistamines [3] and some others [4–6]. The methods are based on the absorbance readings (815 or 750 nm) of intense blue coloured TCNQ$^{\cdot-}$ radical anion formed by interaction of the reagent (a π-acceptor) with n-electron donor drugs in acetonitrile at room temperature.

Rapid detection of antidepressants is important in both clinical and forensic toxicology as patients who have taken overdoses of these drugs are seen frequently. Several methods have been published on the simultaneous chromatographic detection of antidepressants and related drugs. Some methods are based on TLC [7,8] but mostly HPLC [9–13]. As UV detection is used in most existing HPLC methods, the selectivity is limited and somewhat critical. Multicomponent analysis [12] and dual ultraviolet

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wavelength detection [13] have also been used for identification. The present paper describes very selective and rapid HPLC and TLC methods for the simultaneous detection of some antidepressants using TCNQ as a derivatization reagent for the first time. Under certain conditions, TCNQ gave a purple derivative with only aliphatic secondary amine bearing drugs among a vast number of others. The HPLC method is convenient to detect these drugs in plasma whereas the TLC method has a potential to detect them in overdose or forensic cases.
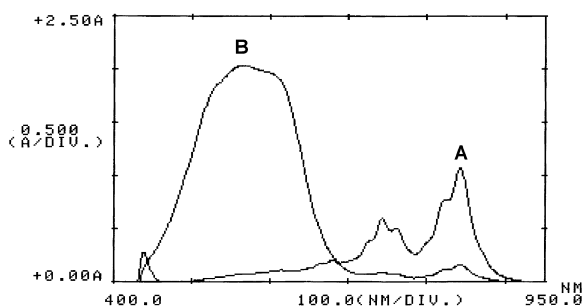

Fig. 1. Absorption spectra of maprotiline-TCNQ reaction products formed (A) at room temperature, (B) after heating at 80 °C for 20 min. Concentration: 20 μg ml^{-1}.

2. Experimental

2.1. Materials and reagents

Fluoxetine hydrochloride (FL), sertraline hydrochloride (SE) and barbexaclone (BA) were kindly provided by Adeka (Istanbul, Turkey), Eczacibasi (Istanbul, Turkey) and Knoll (Istanbul, Turkey), respectively. Paroxetine hydrochloride (PA), 2-hydroxydesipramine and desmethylmaprotiline were a generous gift from Novartis (Basle, Switzerland). Maprotiline hydrochloride (MA), desipramine hydrochloride (DE), nortriptyline hydrochloride (NO) and norfluoxetine were purchased from Sigma (St Louis, MO, USA). TCNQ was from Fluka (Neu-Ulm, Germany). Chemicals and solvents were of analytical or HPLC grade. Bidistilled water was used throughout the assay.

2.2. Solutions

Stock solutions of individual drugs and their mixtures were prepared in water by dissolving their salts giving a concentration of 60 μg ml^{-1} as bases. These solutions were further diluted with water to required concentrations for working solutions. Plasma samples containing known amounts of the drugs were prepared by adding the appropriate volume of these solutions to drug-free plasma. A freshly prepared solution of TCNQ was used at a concentration of 2 mg in 10 ml acetonitrile. A 1 M sodium bicarbonate solution was prepared by dissolving 4.2 g of sodium bicarbonate in 50 ml of water.

2.3. Recording the spectra in Fig. 1

First a solution of maprotiline base was prepared in acetonitrile (100 μg ml^{-1}). To 2 ml of TCNQ reagent, 2 ml of the base solution was added (a pale green colour appeared) and the volume was brought to 10 ml with acetonitrile. In a separate experiment, the same mixture of the reagent and base solution was heated at 80 °C for 20 min (the colour turned purple), cooled and diluted to 10 ml. The spectra of these two solutions (A and B, respectively) were recorded against reagent blanks prepared similarly.

2.4. Sample preparation

Blood samples were collected into EDTA tubes. These tubes were centrifuged (4500 g for 15 min) just after collection and plasma was stored at -20 °C until use.

A 1-ml spiked plasma sample was alkalized with 1 ml of 1 M NaHCO$_3$ solution. The free bases were extracted with 6 ml of hexane–ethylacetate (1:1) by vortex-mixing for 5 min. After centrifugation (4500 g, 5 min), the supernatant was transferred into another tube and Na$_2$SO$_4$ (nonaq.) was added. In a stoppered tube, 5 ml of this solution was evaporated to dryness under nitrogen with mild heating; 100 μl of TCNQ reagent was added to the residue and the mixture was heated at 80 °C for 20 min. For HPLC, 20 μl of sample was directly injected into the column. For TLC, the solvent of the reaction mixture was evaporated to dryness under nitrogen. The residue was dissolved in 20 μl of CHCl$_3$ and the total volume was spotted to the plate with a mi-

cropipette (Desaga, Heidelberg, Germany). The standards were also spotted on the same plate.

2.5. Instrumentation

A Shimadzu UV-160A UV–Visible spectrophotometer was used for absorbance measurements.

The HPLC apparatus and conditions were as follows: a Therma Separation Products liquid chromatograph with Model spectra system®, P 4000 HPLC pump; UV 3000 detector (Therma Separation, TX, USA); ShimPack CLS-ODS column (250×4 mm I.D., 5 μm; Shimadzu, Kyoto, Japan); sample volume, 20 μl; detector was set at 567 nm; mobile phase, acetonitrile–water (60:40); flow-rate, 1.0 ml min^{-1}; run time, 40 min at ambient temperature.

2.6. TLC study

The TLC plates were 10×10 cm polyester plates pre-coated with 0.25 mm Silica Gel 60 (Alltech). The HPTLC plates were 10×10 cm glass plates pre-coated with 0.2 mm Silica Gel F_{254S}CN (Merck). For TLC, the mobile phase was benzene–ether (1:4) (Mobile phase I), for HPTLC, the mobile phases were hexane–ether–acetonitrile (4:4:1) (Mobile phase II), and petroleum ether(40–60°)–ether–acetonitrile–ethyl methyl ketone (5:4:0.5:0.5) (Mobile phase III). Presaturation in developing chambers was carried out with the mobile phases for 30 min. The plates were developed to a distance of 8 cm in the dark. After the development, the plates were air dried in the dark and each R_f value of the purple spots was recorded.

3. Results and discussion

As mentioned earlier, TCNQ has been used only for the spectrophotometric determination of some drugs based on the blue coloured TCNQ$^{.-}$ radical anion. Among the π-acceptors TCNQ has been the reagent of choice, giving the predominant chromogen of the highest molar absorptivity with n-electron donors. Hertler et al. [14] reported that TCNQ also forms substituted quinodimethanes with certain primary and secondary amines, in which one

or two cyano groups of TCNQ are replaced by the amine. If the reagent is excess in the reaction mixture, one cyano group is replaced by the amine forming mostly purple quinodimethanes. Reaction of the purple quinodimethanes or TCNQ itself with excess amines results in the displacement of the two cyano groups with amine yielding mostly yellow or pale yellow products.

In Fig. 1, curve A represents the absorption spectrum of the blue coloured TCNQ$^{.-}$ radical anion formed by the interaction of MA base with TCNQ in acetonitrile at room temperature. When the reaction mixture is heated at 80 °C, one cyano group is replaced by MA by the displacement reaction and a purple coloured substituted quinodimethane is formed. Curve B in the figure shows the absorption spectrum of the purple chromogen of MA. As can be seen from the figure, the quinodimethane has a much higher molar absorptivity value than the TCNQ$^{.-}$ radical anion. For complete reaction, about 20 min of heating is satisfactory for all of the drugs. The structure of the corresponding chromogen of NO was proved by its NMR data in the previous paper [15] that described spectrophotometric determination of NO and DE based on these derivatives. If excess amine is added to the purple solution, the colour turns to yellow suggesting the further displacement of maprotiline with the second cyano group in the molecule. Except that of paroxetine (λ_{max}580 nm), the absorption spectra of the purple chromogens of the drugs (λ_{max}567 nm) are almost the same at the 400–700 nm region. Fig. 2 shows the spectra of three chromogens in this region. Therefore for identification of the drugs, UV spectra of the chromogens must be recorded during the HPLC. As
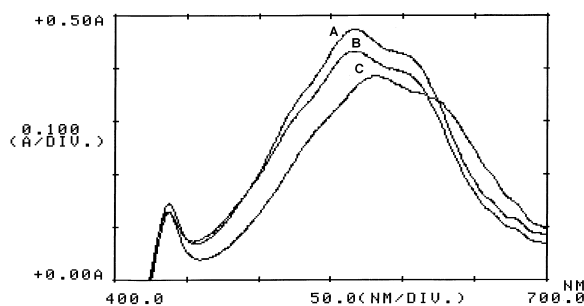

Fig. 2. Absorption spectra of the purple chromogens of fluoxetine (A), maprotiline (B) and paroxetine (C).

can be seen from Fig. 3, these spectra are different enough for definite identification.

The chromogens are very stable when kept in the dark. Addition of water or methanol to the reaction mixture causes slow fading indicating the gradual deterioration in time. It is obvious that acetonitrile and chemicals used for the reaction must be in analytical grade.

For analysis, spiked plasma samples (1 ml) were alkalized with $NaHCO_3$ and the bases liberated were extracted with hexane–ethylacetate (1:1) used by Jedrychhowski et al. [16]. The solvent was evaporated with a stream of nitrogen and excess reagent in
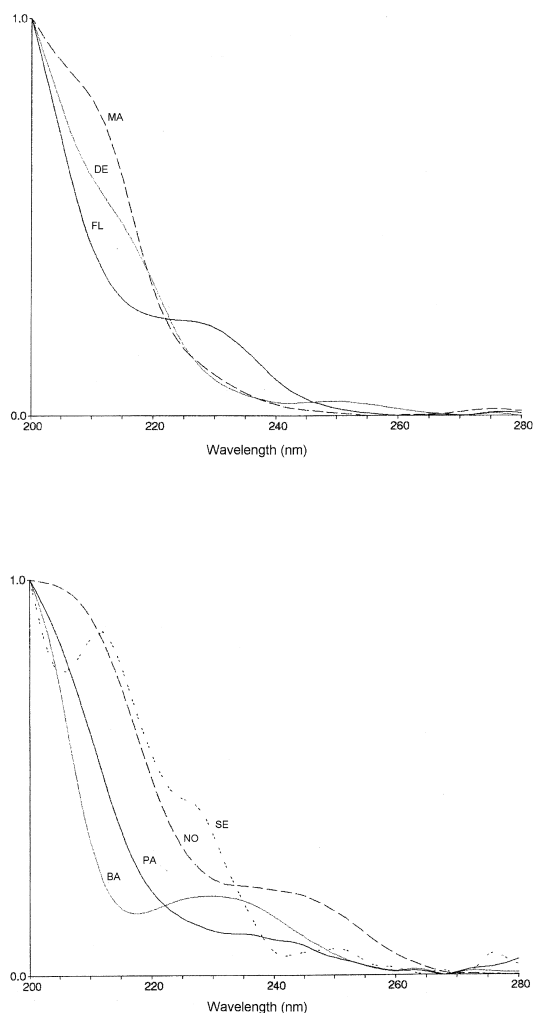
acetonitrile (100 µl) added to the residue. The mixture heated at 80 °C for 20 min. Without any sample clean-up, 20 µl of the reaction mixture was directly injected to a C_{18} column for HPLC separation. Acetonitrile–water (60:40) was used as the mobile phase for isocratic elution at ambient temperature. The flow-rate was 1 ml min^{-1}, and the detector was set at 567 nm (for paroxetine alone, 580 nm). As can be seen from Fig. 4B, a satisfactory separation was obtained in a 40-min run. Although the derivatives of FL and DE were incompletely separated from each other, the separation is still sufficient for detection of these drugs. If another antidepressant, sertraline (SE) is present in the sample it interferes with the detection of NO at a minimum concentration of 500 ng ml^{-1}. Because under the reaction conditions, SE forms a small amount of a purple derivative and much of the TCNQ$^{·-}$ radical anion remains in the reaction mixture. As can be seen from Fig. 5A, these two derivatives are well separated by TLC. If antiepileptic BA is present, it interferes with the detection of PA both by HPLC and TLC but they are well separated by HPTLC (Fig. 5B). Fig. 4C shows that 36 ng ml^{-1} concentration of the drugs gives easily detectable peaks.

Although TCNQ$^{·-}$ radical anion was formed by interaction of TCNQ with the metabolites 2-hydroxy-desipramine, desmethylmaprotiline and norfluoxetine, no purple derivative was produced on heating. This was expected for norfluoxetine and desmethylmaprotiline because in our preliminary experiments, primary aliphatic amine-bearing drugs such as mexiletine, amlodipine and fluvoxamine failed to form a purple derivative with TCNQ under described reaction conditions.

By the proposed HPLC method, in analyses of plasma samples from patients or a volunteer receiving fluoxetine, paroxetine or maprotiline, we observed only a single peak in the chromatogram having the same retention time as the derivative of the parent drug. The chromatogram of fluoxetine is shown in Fig. 4D.

For TLC separation, the whole reaction mixture was applied to the plates after concentrating. Three solvent systems were used (Mobile phase I for TLC; II and III for HPTLC). As can be seen from Fig. 5 (in order to obtain satisfactory photographs of the

Fig. 3. UV spectra of the chromogens of the five antidepressants, sertraline and barbexaclone.
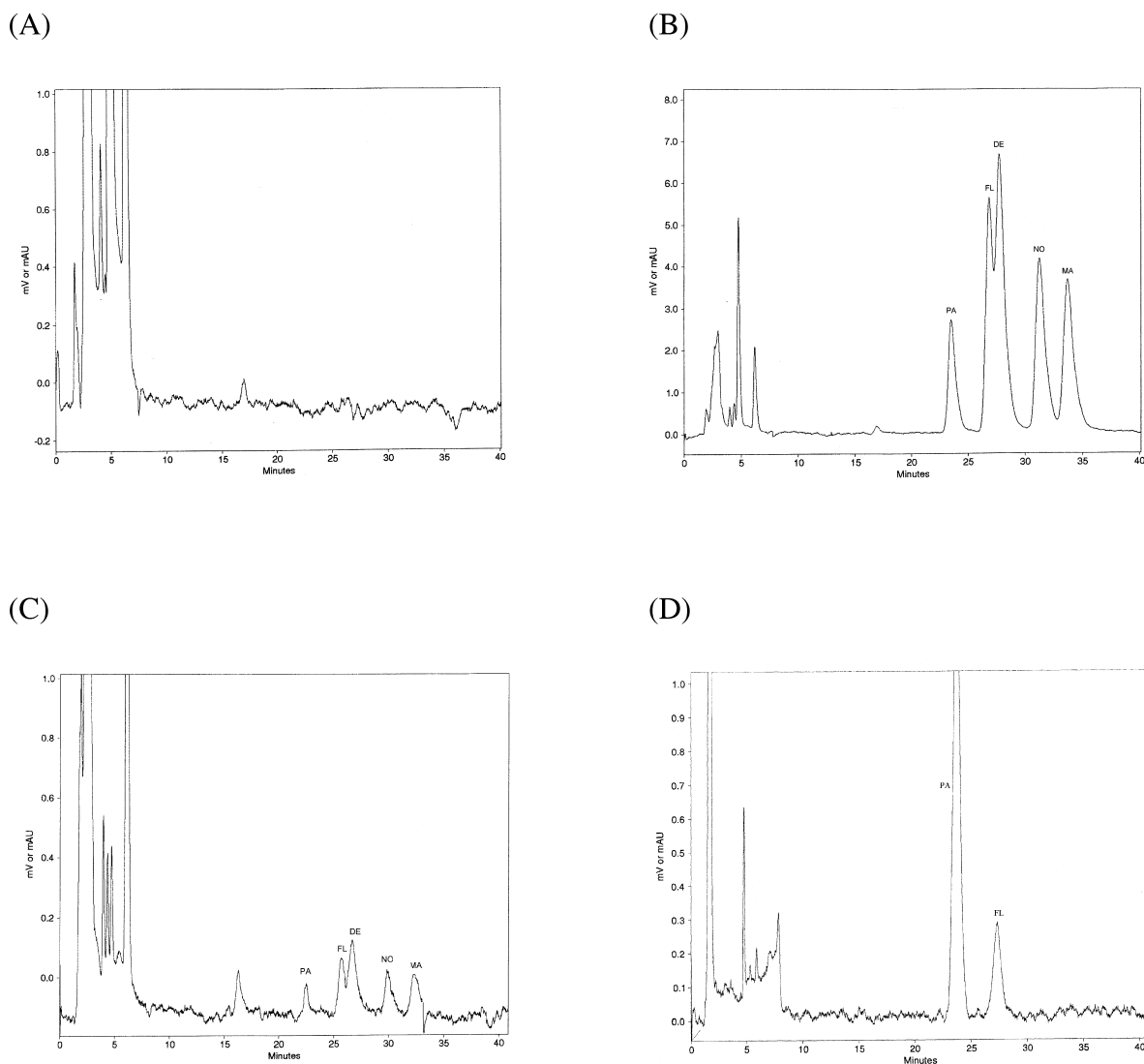
(A)

(B)

(C)

(D)

Fig. 4. Chromatograms of the purple chromogens of the antidepressants from analyses at 567 nm (A), blank plasma (B) and (C) spiked plasma samples containing 600 and 36 ng ml^{-1} of each drug, respectively. (D) A plasma sample taken 1 h after a 20 mg oral dose of FL. PA is the internal standard (200 ng ml^{-1}).

plates in Fig. 5, much higher concentrations of the compounds were used for these separations), the best separation was achieved by TLC. Only the derivatives of NO and DE are not separated, similar to on HPTLC plates, but they are well separated by HPLC. R_f and R_{std} (relative to maprotiline) values are listed in Table 1. Standard deviations in all R_f values are less than 0.02. Detection limits (Table 2) on TLC were found lower than on HPTLC probably due to

spot broadening on HPTLC. Table 2 indicates that detection limits are 240–480 and 480–720 ng ml^{-1} plasma by TLC and HPTLC, respectively. For PA, the limits are higher being 1.20 and 1.50 µg ml^{-1}, respectively.

The main advantage of the proposed methods over the previously published methods is their higher selectivity. A number of drugs do not interfere with the detection due mostly to the lack of purple
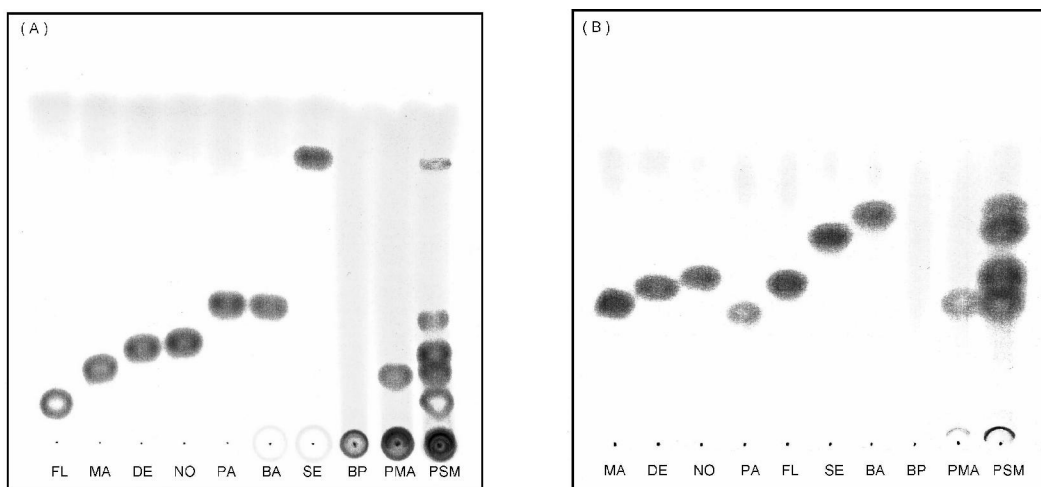
Fig. 5. Separation of the purple chromogens of the antidepressants and barbexaclone (A) on TLC (Mobile phase I) and (B) HPTLC (Mobile phase III), where BP is blank plasma; PMA and PSM, plasma spiked with maprotiline and standard mixture, respectively.

Table 1
R_f and R_{std} values of substances for the three systems

Substance	TLC data Mobile phase I		HPTLC data Mobile phase II		Mobile phase III	
	R_f^a	R_{std}^b	R_f^a	R_{std}^b	R_f^a	R_{std}^b
Maprotiline	19	1.00	27	1.00	37	1.00
Desipramine	26	1.37	29	1.07	41	1.11
Nortriptyline	27	1.42	32	1.19	44	1.19
Paroxetine	37	1.95	24	0.89	34	0.92
Fluoxetine	10	0.53	31	1.15	42	1.14
Barbexaclone	36	1.89	42	1.56	61	1.65
Sertraline	74	3.89	38	1.41	54	1.46

[a] $R_f \times 100$ (average of four runs).
[b] R_{std} relative to maprotiline (standard).

Table 2
Detection limits of substances ($\mu g\ ml^{-1}$ plasma) by TLC and HPTLC

Substance	TLC	HPTLC
Maprotiline	0.36	0.60
Desipramine	0.36	0.60
Nortriptyline	0.36	0.60
Paroxetine	1.20	1.50
Fluoxetine	0.24	0.48
Barbexaclone	0.48	0.72
Sertraline	0.36	0.60

derivative or elimination at the extraction stage. Among those are some primary amines, primary and secondary amino acids and even some secondary amines. It is obvious that tertiary amines and phenols do not give any reaction with TCNQ. Fig. 4A shows that from the endogenous plasma constituents only one peak arises in HPLC but it is quite far from the main peak area. On TLC or HPLC plates no purple spot was observed from endogenous compounds. Antiepileptic barbexaclone does interfere with the detection of paroxetine by HPLC but the chromogens

A. Oztunc et al. / J. Chromatogr. B 774 (2002) 149–155

155

are well separated by TLC. Moreover the described methods are simple. No buffer solution is used, and no prior sample clean-up is required. The HPLC method was applied to analyses of real plasma samples and satisfactory results were obtained. Being very selective, this method can also serve a laboratory as a means of confirming a presumptive identification of these antidepressants by another method. The TLC method is suitable for qualitative analysis of these drugs and has a potential for rapid and economic toxicologic and forensic screening of these drugs.

Acknowledgements

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