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DEVELOPMENT OF A NEW MICRO-HPTLC PROTOCOL FOR TOTAL ANTIOXIDANT POTENTIAL DETERMINATION OF REDOX-ACTIVE DRUGS

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ABSTRACT. A new micro-HPTLC method assisted by image analysis technique was proposed for rapid evaluation of the total antioxidant potential (TAP) of redox-active drugs using 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and 2,2'-azinobis-3 ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$) radicals. Colored compounds that can significantly influence the spectrophotometric and chromatographic measurements were revealed in case of selected drugs after their reaction with DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radical respectively. The best chromatographic conditions were selected for accurate quantification of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals after their separation from the interfering compounds. The validation of the proposed method was performed in terms of linearity, limit of detection, limit of quantification, precision, and accuracy. Green and red color channel used for image processing were found to allow accurate quantification of DPPH$^{\bullet}$. For accurate quantification of ABTS$^{\bullet+}$ the red and gray channels were selected. A linear dependence of spot area/radical concentration was obtained in the range 0.78-2.76 μg·spot^{-1} and 0.21-2.49 μg/spot for DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals respectively. The developed method was found to be accurate and precise according to values higher than 92% for recovery and less than 3% for relative standard deviation parameters (RSD%). A statistical significant correlation was obtained comparing the spectrophotometric results with the chromatographic ones obtained for selected adrenergic drugs.

Keywords: *micro-HPTLC method; image analysis; total antioxidant potential; adrenergic drugs; DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ protocols*

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INTRODUCTION

The development of rapid protocols to measure the antioxidant activity of different classes of compounds is one of the topics with a great scientific interest in recent years [1-3]. High performance thin-layer chromatography combined with image analysis (HPTLC-IA) method is among the recently developed methods in the area of the antioxidant potential evaluation. Protocols based on scavenging the free-radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) were found to be mostly used for the *in vitro* antioxidant activity evaluation purpose [4-9]. In the most popular approaches the typical planar chromatographic assay is used. In this approach developed plates are sprayed or immersed with/in DPPH$^{\bullet}$ radical solution and the active antiradical constituents (appeared as yellowish white spots produced by bleaching the purple color of the DPPH• reagent) are quantified based on the integrated area of the chromatographic spot [5].

The HPTLC-IA methodology has the advantage of multiple parallel sample analysis on the same chromatographic plate, possibility to present results as a colorful image, multiple levels of visualization (in white light, UV 366 nm and/or UV 254 nm) and moreover the possibility of application of advanced image analysis techniques. The use of different color scale (channel) selection for image processing provides complementary information and allow an accurate quantification of compounds even if they are not separated with high resolution from the matrix constituents. Based on sensitive and accurate protocols developed until now, the HPTLC-IA method represents a promising, rapid and economic alternative for comprehensive evaluation of the antioxidant potential of classes of redox-active compounds. Results published in prestigious international journals [7-10] sustain the HPTLC-IA method as promising, rapid and economic alternative able to provide accurate evaluation/quantification of antioxidant potential of redox-active compounds and investigate/monitor the reaction mechanism with DPPH• radical.

In the micro-thin-layer chromatographic assay (micro-TLC) the antioxidant potential is evaluated by quantitative analysis of radical DPPH$^{\bullet}$ and DPPH-H molecules after reaction with the sample [11], and then separated from the interfering compounds. So, the micro-TLC approach can offer the possibility of evaluation of total antioxidant potential (TAP) of redox-active compounds in free-of-interaction liquid medium. Combined with image analysis procedures by color scale selection, the micro-TLC method provides complementary information that may allow accurate quantification of radical molecules even if they are not well separated from the sample matrix constituents.

The objective of this study was to develop a micro-HPTLC method assisted by image analysis techniques (micro-HPTLC-IA method) for accurate determination of total antioxidant potential (TAP) of redox-active drugs

using both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$radicals. During the method development, the chromatographic conditions were optimized and different image analysis techniques were evaluated for accurate determination of both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals. The method was validated in terms of linearity range, limit of determination and limit of quantification and the method advantages were highlighted for the determination of the antioxidant potential of selected adrenergic drugs.

RESULTS AND DISCUSSION

Optimization of the chromatographic parameters for the micro-HPTLC method

The preliminary investigations for selected adrenergic drugs using typical chromatographic method (TLC) revealed a considerable antiradical activity of these drugs against DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals (Figure 1).

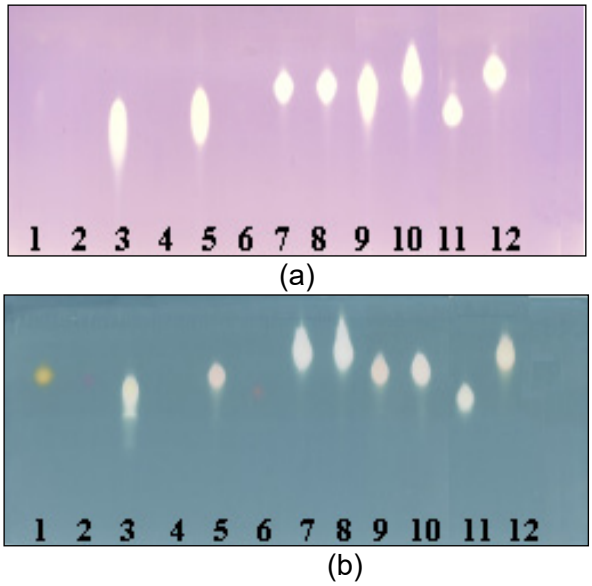

Figure 1. Image of the chromatographic plate (HPTLC Silica gel 60 F_{254}) after separation of selected adrenergic drugs (mobile phase methanol: water: formic acid, 60:40:0.5 v/v/v) and immersion in DPPH$^{\bullet}$ solution ($0.8x10^{-3}$ mol/L) (a) and ABTS$^{\bullet+}$ solution ($0.7x10^{-3}$moli/L) (b). (1-Terbutaline; 2-Albuterol; 3-Adrenalone; 4-Methoxamine; 5-Isoprenaline; 6-Ritodrine; 7-L-DOPA; 8-D-DOPA; 9-Epinephrine; 10-Norepinephrine; 11- Caffeic acid; 12-Trolox)

During our investigations, for some drugs as terbutaline (1), albuterol (2) and ritodrine (6) the reaction with $ABTS^{\bullet+}$ radical on the chromatographic plate conducted to the formation of colored compounds (yellow-orange for terbutaline; purple-red for albuterol; pink-red for ritodrine) that can significantly influence the spectrophotometric and typical chromatographic measurements. Thus, for these drugs, the evaluation of the antioxidant potential using the classical spectrophotometric and typical chromatographic methods cannot be carried out with precision for the $ABTS^{\bullet+}$ assay. For these situations the micro-TLC approach offer a promising alternative for rapid evaluation of total antioxidant potential (TAP) [11].

Development of the proposed micro-HPTLC protocol for quantitation of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals started from optimization of the chromatographic conditions necessary for accurate determination of these radicals.

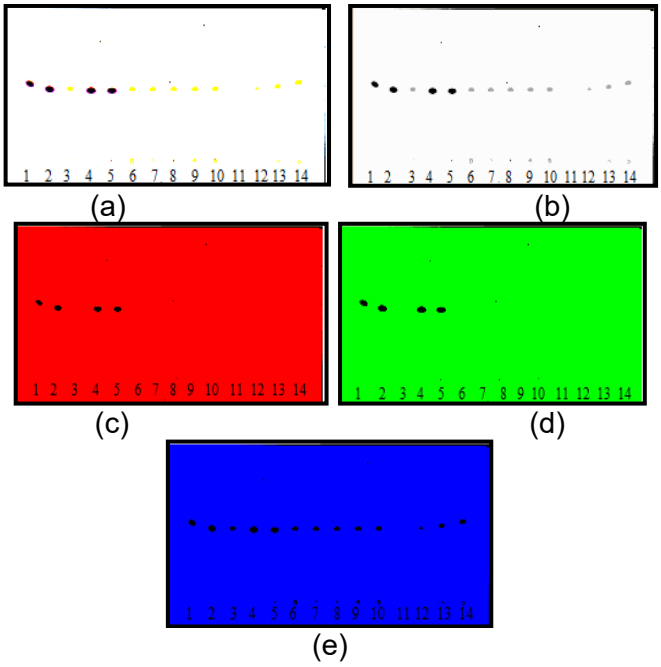

Figure 2. Image of the chromatographic plate (HPTLC Silica gel 60 F_{254s}) obtained after separation of $DPPH^{\bullet}$ radical molecules and image processing using the ImageDecipher TLC software: (a) RGB (red, green and blue primary colors) color mode; (b) gray scale selection; (c) red scale selection; (d) green scale selection; (e) blue scale selection. (1-Terbutaline, 2-Albuterol, 3-Adrenalone, 4-Metoxamine, 5-Ritodrine, 6-Isoprenaline, 7-L-DOPA, 8-D-DOPA, 9-Epinephrine, 10-Norepinephrine, 11,12,13,14-Caffeic acid -2,4,6 and 8 µL).

In order to determine the optimal conditions of separation of DPPH• and DPPH-H, different stationary phases and mobile phases were tested. For the HPTLC Silica gel 60 F_{254s} plates mixtures of hexane and acetone (50:50; 60:40 and 70:30 v/v) and hexane, acetone and ethanol (60:30:10; 60:35:5 and 60:38:2 v/v/v) were used. Also diferent mixtures of methanol-water (from 40% to 70% methanol) were tested using the Silica gel 60 RP-18W F_{254s}, HPTLC Silica gel 60 RP-8 F_{254s} and HPTLC Silica gel 60 DIOL F_{254s} plates. The best results were obtained for HPTLC Silica gel 60 F_{254s} plates with mobile phase consisted of hexane: acetone: ethanol (60:38:2 v/v/v). In these conditions, both forms of DPPH (DPPH$^{\bullet}$ and DPPH-H) were not completely separated but selective quantification of DPPH$^{\bullet}$ molecules was performed in red and green channel respectively.

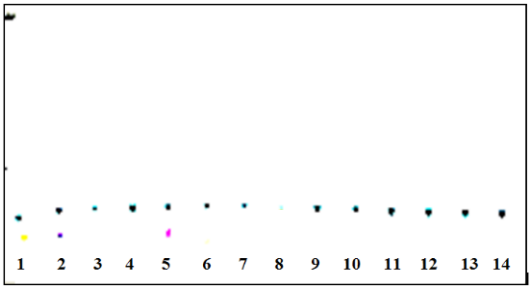

Figure 3. RGB (red, green and blue primary colors) image of the chromatographic plate (HPTLC silica gel RP-18W F_{254s}) obtained after separation of ABTS$^{\bullet+}$ radical molecules and image processing using the ImageDecipher TLC software. (1-Terbutaline; 2-Albuterol; 3-Adrenalone; 4-Metoxamine, 5-Ritodrine, 6-Isoprenaline, 7-L-DOPA, 8-D-DOPA, 9-Epinephrine; 10- Norepinephrine; 11, 12, 13, 14 - ABTS$^{\bullet+}$ sample).

For the ABTS assay the optimal condition for separation of ABTS$^{\bullet+}$ radical (green spots) from the colored interfering constituents (resulted from reaction of terbutaline, albuterol and ritodrine respectively with ABTS$^{\bullet+}$ radical) was targeted. The best results (Figure 3) were obtained for HPTLC silica gel RP-18W F_{254s} plates and mixture of methanol: water (40:60 v/v) as mobile phase. The gray and red channels provided the best sensitivity for ABTS$^{\bullet+}$ spots and both channel were selected to perform quantification of ABTS$^{\bullet+}$ molecules.

Method Validation

The method performance parameters expressed as linearity range, limit of detection (LOD) and limit of quantification (LOQ) for developed micro-HPTLC-DPPH$^{\bullet}$ and micro-HPTLC-ABTS$^{\bullet+}$ protocols are presented in Table 1.

Table 1. Method performance parameters (linearity range, limit of detection - LOD and limit of quantification - LOQ) obtained for developed micro-HPTLC- DPPH$^{\bullet}$ and micro-HPTLC-ABTS$^{\bullet+}$ assays.

Metod performance parameters	micro-TLC- DPPH$^{\bullet}$ assay		micro-TLC- ABTS$^{\bullet+}$ assay	
Calibration range	0.78 - 2.76 (μg/spot)		0.21 - 2.49 (μg/spot)	
	Red channel	Green channel	Gray channel	Red channel
Intercept	-1254.10	480.89	57.80	121.81
Slope	2412.60	2166.70	3884.30	5430
*R^2	0.9881	0.9918	0.9884	0.9946
LOD (µg/spot)*	0.24±0.01	0.22±0.01	0.03±0.01	0.02±0.01
LOQ (µg/spot)*	0.79±0.02	0.73±0.01	0.10±0.02	0.07±0.02

*regression determination coefficient ; *values ±standard deviation (SD)

A good linear dependence ($0.9881 < R^2 < 0.9946$ for spot area of radical vs. radical concentration) was obtained for 0.78 - 2.76 (µg/spot) and for 0.21 - 2.49 µg/spot in case of micro-HPTLC-DPPH$^{\bullet}$ and micro-HPTLC-ABTS$^{\bullet+}$ assay, respectively. Green channel selection was revealed to offers a better sensitivity (LOD = 0.22 µg/spot) for DPPH$^{\bullet}$ quantification while the red channel selection conducted to beter sensitivity for ABTS$^{\bullet+}$ determination (LOD = 0.02 µg/spot).

The accuracy of the micro-HPTLC method was evaluated by quantification of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ molecules on three concentration levels of radical /spot. Results, expressed by percent recovery ranged from 92% to 96% for DPPH$^{\bullet}$ and from 0.93% to 95% for ABTS$^{\bullet+}$ radical, respectively.

The relative standard deviation (RSD%) values less than 3% obtained by analysis of replicates of radical solution at three different concentrations (for both radicals) during three different time in one day revealed a good intraday precision and good repeatability of the method.

Application of the developed micro-HPTLC method for the determination of total antioxidant potential (TAP) of selected adrenergic drugs

Based on method development results, the total antioxidant potential for selected adrenergic drugs (Table 2) was determined by micro-HPTLC-DPPH$^{\bullet}$ assay using red and green channels selection and by micro-HPTLC-ABTS$^{\bullet+}$ assay using gray and red channels selection respectively. Results were expressed as caffeic acid equivalents for the DPPH$^{\bullet}$ method and trolox equivalents for the ABTS$^{\bullet+}$ method (calibration curves parameters for reference antioxidants caffeic acid in DPPH$^{\bullet}$ assay and trolox in ABTS$^{\bullet+}$ assay: $0.9990 < R^2 < 0.9996$).

The obtained results revealed that catecholamine related drugs (L-DOPA, D-DOPA, adrenalone, epinephrine and isoprenaline) show a high antiradical potential against $DPPH^{\bullet}$. Ritodrine and terbutaline showed comparable activity with norepinephrine.

Similar results (expressed in trolox equivalents) were obtained by micro-HPTLC-$ABTS^{\bullet+}$ assay. L-DOPA, D-DOPA, adrenalone and isoprenaline (catecholamine related drugs) were revealed as drugs with highest antioxidant potential. They are followed by terbutaline, norepinephrine, albuterol and ritodrine.

Table 2. Total antioxidant potential (TAP) for selected adrenergic drugs determined by micro-HPTLC- $DPPH^{\bullet}$ and micro-HPTLC-$ABTS^{\bullet+}$ assays.

Sample No.	Sample name	Total antioxidant potential (TAP)			
		micro-TLC-$DPPH^{\bullet}$ (caffeic acid equivalents*) (μM)		micro-TLC-$ABTS^{\bullet+}$ (trolox equivalents*) (μM)	
		Red channel	Green channel	Gray channel	Red channel
1	Terbutaline	71.46±0.16	71.99±0.58	58.28±1.01	55.99±1.02
2	Albuterol	25.63±0.27	24.74±2.82	44.28±0.98	43.66±1.51
3	Adrenalone	153.83±1.27	150.24±1.84	85.41±1.41	87.53±1.06
4	Metoxamine	6.43±0.04	7.09±0.12	0.72±0.20	0.88±0.08
5	Ritodrine	74.49±0.18	78.62±1.05	29.58±1.58	28.55±1.97
6	Isoprenaline	124.34±1.51	126.27±0.03	69.62±1.21	72.10±0.13
7	L-DOPA	168.39±1.27	163.69±0.88	142.06±1.44	144.69±0.78
8	D-DOPA	154.83±2.11	152.28±1.66	88.67±2.04	84.46±2.98
9	Epinephrine	136.72±0.20	134.94±1.44	18.51±0.76	17.38±1.26
10	Norepinephrine	73.14±1.10	75.05±1.31	48.21±1.08	50.80±1.54

*±SD - standard deviation determined for two replicate spots

Evaluation of the chromatographic results obtained by developed micro-HPTLC-$DPPH^{\bullet}$ and micro-HPTLC-$ABTS^{\bullet+}$ assay respectively was made by comparison with results from classic spectrophotometric assay using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals. For this purpose, the antioxidant activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals was determined for the studied compounds using the spectrophotometric method. The spectrophotometric results expressed as caffeic acid equivalqnts (μM) for $DPPH^{\bullet}$ assay and as trolox equivalents (μM) for $ABTS^{\bullet+}$ assay were calculated based on the calibration curves (% of consumed radical vs. concentration of the reference antioxidant) obtained for the reference antioxidants caffeic acid ($y = 204.86x + 1.9056$, $R^2 = 0,9999$) and trolox ($y = 3.3107x + 2.5715$, $R^2 = 0,9998$), respectively. The obtained results for the selected drugs are presented in Table 3.

Table 3. Total antioxidant potential (TAP) for selected adrenergic drugs determined by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ spectrophotometric assays.

No.	Sample name	Total antioxidant potential (TAP)	
		$DPPH^{\bullet}$ assay Caffeic acid equivalents* (μM)	$ABTS^{\bullet+}$ assay Trolox equivalents* (μM)
1	Terbutaline	70.42±0.14	10.31±0.15
2	Albuterol	35.06±0.07	47.3±0.13
3	Adrenalone	140.17±0.15	85.81±0.18
4	Metoxamine	5.25±0.02	0.86±0,08
5	Ritodrine	83.40±0.12	14.81±0.13
6	Isoprenaline	116.41±0.13	74.22±0.14
7	L-DOPA	122.51±0.12	149.50±0.16
8	D-DOPA	125.24±0.16	86.36±0.15
9	Epinephrine	138.75±0.18	19.20±0.12
10	Norepinephrine	50.88±0.13	49.71±0.11

*±SD - standard deviation determined for three replicate measurements of absorbance

For L-DOPA, D-DOPA and adrenalone drugs, the spectrophotometric results showed a similar behavior as in the chromatographic assay, showing a high activity against both the $DPPH^{\bullet}$ and the $ABTS^{\bullet+}$ radicals. Contrary to the chromatographic determinations, the spectrophotometric assay indicated a considerable activity for terbutaline and ritrodrine against the $ABTS^{\bullet+}$ radical. These high values clearly highlight that the spectrophotometric determination is affected by the presence of intensely colored compounds formed from the reaction of these drugs with the $ABTS^{\bullet+}$ radical, compounds that were revealed by typical chromatographic investigations (Figure 1) and also by the developed micro-HPTLC method (Figure 3).

Table 4. The correlation parameters obtained between chromatographic and spectrophotometric results

Method *	DPPH (1)	DPPH (2)	ABTS (1)	ABTS (2)	DPPH (3)	ABTS (3)
DPPH (1)	1.00	**1.00**	**0.73**	**0.72**	**0.96**	**0.72**
DPPH (2)	**1.00**	1.00	**0.72**	**0.71**	**0.96**	**0.71**
ABTS (1)	**0.73**	**0.72**	1.00	**1.00**	0.57	**0.94**
ABTS (2)	**0.72**	**0.71**	**1.00**	1.00	0.56	**0.94**
DPPH (3)	**0.96**	**0.96**	0.57	0.56	1.00	0.55
ABTS (3)	**0.72**	**0.71**	**0.94**	**0.94**	0.55	1.00

* DPPH (1): micro-HPTLC-DPPH (red channel); DPPH (2): micro-HPTLC-DPPH (green channel); ABTS (1): micro-HPTLC-ABTS (gray channel); ABTS (2): micro-HPTLC-ABTS; DPPH (3): DPPH spectrophotometric assay; ABTS (3): ABTS spectrophotometric assay.

The comparison of the obtained results (Table 4) showed a significant correlation between the spectrophotometric and developed micro-HPTLC-DPPH$^{\bullet}$ method on both red channel and green channel selection analysis (r = 0.96 in both cases).

Also in case of micro-HPTLC-ABTS$^{\bullet+}$ assay a significant correlation was observed with spectrophotometric results for both channel selected (r = 0.94) In this case a significant increasing of the correlation parameter was obtained (r > 0.99) when terbutaline and ritodrine were not considered for correlation study.

CONCLUSIONS

The micro-HPTLC method using the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals can be succesfully applied for accurate determination of total antioxidant potential of redox-active drugs. The use of HPTLC Silica gel 60 F_{254} plates with a mobile phase consisted of hexane: acetone: ethanol (60:38:2 v/v/v) and green channel selection allow an accurate quantification of DPPH$^{\bullet}$ radical. New compounds were revealed after reaction of ABTS$^{\bullet+}$ radical with selected adrenergic drugs (terbutaline, albuterol and ritodrine respectively). The best separation of these compounds from the ABTS$^{\bullet+}$ molecules was obtained using the HPTLC silica gel RP-18W F_{254} plates and mixture of methanol: water (40:60 v/v) as mobile phase. The validation of the micro-HPTLC-DPPH and micro-HPTLC-ABTS protocols performed in accordance with ICH guidelines indicates that the developed micro-HPTLC-IA method meets the criteria for linearity, precision and accuracy. In the above chromatographic conditions, a significant correlation (r > 0.90) was revealed between chromatographic and reference spectrophotometric results obtained for selected adrenergic drugs. It can be concluded that the proposed micro-HPTLC-IA method involving both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals is a powerful tool that allow a rapid evaluation of total antioxidant potential of redox-active drugs. The most significant advantage of the developed method is their accurate quantification of radicals even in presence of interfering compounds that can be formed by reaction of radicals with redox-active compounds.

EXPERIMENTAL SECTION

Reagents and samples

High purity standards of terbutaline, albuterol, adrenalone, methoxamine, ritodrine, isoprenaline, L-DOPA, D-DOPA, epinephrine and norepinephrine drugs and caffeic acid and trolox reference antioxidants were purchased from

Sigma-Aldrich company (Steinheim, Germany) and used for samples preparation in this study. Standards of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2'-azinobis-(3-ethylbenzothiazole-6-sulfonic acid) (ABTS) and potassium persulfate (Sigma-Aldrich, Steinheim, Germany) were used for the preparation of the radical solutions.

Stock solutions of the samples were prepared in ethanol at concentrations of $5x10^{-3}$ mol/L.

Instrumentation and software programs

Linomat V TLC auto-sampler (CAMAG, Muttenz, Switzerland) and a Hamilton syringe (100 µL) were used for sample application on the chromatographic plates. A specialized UV-Vis TLC scanner device (Thin Layer Chromatography Scanner TLC-2400S, BioDit Technology, Co.), the second-generation instrument for quantitative measurements in TLC, equipped with high qualified Micortek 3-linear color CCD camera was used for image acquisition.

Jasco V550 computer controlled spectrophotometer (Tokyo, Japan) equipped with double beam, single monochromator and quartz cuvettes of 1cm optical path was used for spectral measurements.

ImageDecipher software (BioDit Technology, Co.) was used for image processing and analysis.

Spectra Manager for Windows 95/ NT version 1.53.04 (1995–2002, Jasco Corporation) software package was used for the spectra acquisition control, smoothing process, storage and spectral data digitization.

Statistica 8.0 (StatSoft, Inc. 1984–2007, Tulsa, USA) software was used to perform correlation of the results obtained by the spectrophotometric and micro-HPTLC methods.

Solutions and samples preparation

The DPPH$^{\bullet}$ radical stock solution (concentration$1x10^{-3}$ mol/L) was prepared by dissolving appropriate required amount of DPPH in methanol for both micro-TLC and spectrophotometric measurements. This solution was prepared daily and protected from light throughout the analysis time in order to minimize the loss of free radical activity. Working solutions of different concentrations were prepared by rigorous dilution from stock solution in all cases.

The ABTS$^{\bullet+}$radical stock solution (concentration $1.25x10^{-3}$ mol/L) was produced according to the Ozcan protocol [12] by reacting equal volumes of the ABTS stock solution of 7 mmol/L concentration with solution of 2.45 mmol/l potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 – 16 h before use. The working solutions of ABTS$^{\bullet+}$ radical were prepared by required dilution in methanol.

Stock solutions ($5x10^{-3}$ mol/L concentration) of reference antioxidants and drug samples were prepared by dissolving appropriate required amount of standard in methanol.

The method validation protocol

The proposed method was validated in terms of linearity, precision, accuracy, quantification limit (LOQ) and detection limit (LOD) in accordance with established International Conference on Harmonization (ICH) guideline [13].

Linearity test of developed micro-HPTLC method was made by the dilution of the stock solution of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ respectively to different required concentrations. For this aim a series of ten solutions from $1x10^{-4}$ to $2x10^{-3}$ mol/L concentration of $DPPH^{\bullet}$ and from $0.05x10^{-3}$ mol/L to $1.25x\ 10^{-3}$ mol/L ($\varepsilon = 1{,}6 \times 10^4\ M^{-1}\ cm^{-1}$) $ABTS^{\bullet+}$ were prepared by appropriate dilution in methanol. Eight µL of each solution was spotted on HPTLC plate in all cases. The plates were developed using a mobile phase consisting hexane: acetone: ethanol (60: 38: 2 v/v) for $DPPH^{\bullet}$ and of methanol : water 40 : 60 v/v) for $ABTS^{\bullet+}$separation. After separation, the plates were dried in dark conditions for ten minutes and scanned. All the process analysis was repeated three times. In both cases of radicals the calibration curve was plotted considering peak area versus concentration of radical (μg/spot). The linearity performance parameters for the calibration (coefficient of linear regression (R^2), slope, and intercept) were reported.

The limit of detection (LOD) and limit of quantification (LOQ) values were determined on the basis of specific calibration curves obtained for $DPPH^{\bullet}$ and for $ABTS^{\bullet+}$ respectively. The LOD was calculated as LOD =$3\sigma/S$ and LOQ was calculated as LOQ =10σ/s where σ is the standard deviation of the spot area and S is the slope of the calibration curve.

The accuracy of the method was determined for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assay on three concentration levels of radical/spot. Results were expressed as percent recovery calculated based on spot area of $DPPH^{\bullet}$ respectively $ABTS^{\bullet+}$. In case of micro-HPTLC-$DPPH^{\bullet}$ assay the percent recovery was calculated also for three concentration levels of $DPPH^{\bullet}$ in presence of DPPH-H molecules. These analyses were performed six times in each case.

Repeatability (intraday precision) of the method was determined by the analysis of three replicates of radical solution at three different concentrations of radicals in each case. All solutions were prepared independently and 8µL of were applied on the chromatographic plate in each case. Precision was determined on the basis of measurements of spots area of three different concentration of radical and expressed as the relative standard deviation (RSD%) values.

Chromatographic protocol for TAP measurement

The total antioxidant potential (TAP values) of the drugs under study was determined by developed micro-HPTLC method.

For the DPPH$^{\bullet}$ protocol, 5 mL of DPPH$^{\bullet}$ solution ($1x10^{-3}$ mol/L) were mixed with 50 µL of drug sample (concentration $1x10^{-3}$ mol/L^{-1}) and kept in dark conditions for a 30 minutes reaction time. Spots of eight µL from the each resulted sample were applied as spots on HPTLC Silica gel 60 F_{254} plate (20 cm × 10 cm, Merck, Darmstadt, Germany) with an application speed of 80 nL/s. Development of the plate was carried at room temperature over a distance of 8 cm in a normal chromatographic chamber using the mixture of hexane: acetone: ethanol in the ratio of 60: 38: 2 (v/v) as mobile phase. After separation the plates were dried in dark conditions at room temperature for 10 minutes. Plates were scanned and the quantification of DPPH$^{\bullet}$ was performed based on spot area determined by gray, green, blue and red scales selection. Results were were expressed as caffeic acid equivalents (µg/spot) based on the equation of the calibration line obtained in the same chromatographic conditions for caffeic acid in the concentration range 0.01 - 0.1 µg/spot.

For the ABTS$^{\bullet+}$ protocol, 2 mL ABTS$^{\bullet+}$ solution (concentration $1.25x10^{-3}$ mol/L) were mixed with 50 µL of drug sample solution (concentration $1x\ 10^{-3}$ mol/L) and kept in dark conditions for 30 minutes (reaction time). From each of the resulted samples, spots of 8 µL were applied on the HPTLC silica gel RP-18W F_{254} plates with an application speed of 60 nL/s. Development of the plate was carried at room temperature over a distance of 8 cm in a normal chromatographic chamber using mixture methanol: water in a various proportion (from 20% methanol to 60% methanol (v/v)) as mobile phase. After separation the plates were dried in dark conditions at room temperature for ten minutes and scanned. Quantification of ABTS$^{\bullet+}$ was performed based on spot area determined by gray, green, blue and red scales selection. Results were expressed as trolox equivalents (µg/spot) based on the equation of the calibration curve obtained in the same chromatographic conditions for trolox in the concentration range 0.001 - 0.122 µg/spot.

In all cases the samples were prepared in duplicate and from each sample duplicate spots were applied on the chromatographic plate.

The Spectrophotometric protocol for TAP measurements

For the spectrophotometric assay stock solutions of DPPH$^{\bullet}$ (concentration $1x10^{-3}$ mol/L) and ABTS$^{\bullet+}$ (concentration $1.25x10^{-3}$ mol/L) radicals prepared as described above were used after appropriate dilution in methanol. Working solution of DPPH$^{\bullet}$ of $0.15x10^{-3}$ mol/L was freshly prepared

for all determinations. 5 mL of working solution of $DPPH^{\bullet}$ (concentration $0.15x10^{-3}$ mol/L) was mixed with 50 µL of sample (concentration $1x10^{-3}$ moli/L^{-1}) and the absorbance value was measured at λ=518 nm wavelength after a reaction time of 30 minutes (in dark conditions). Results were expressed as caffeic acid equivalents.

The $ABTS^{\bullet+}$ radical stock solution, prepared as described above, was diluted to an absorbance value of A = 0.700 (for λ=734 nm wavelength corresponding to the maximum of the absorbance; solution concentration 0.044 x 10^{-3} mol/L, calculated based on Lambert Beer's law for a value of the extinction coefficient ε = $1.6x10^{4}$ M^{-1} cm^{-1}) and used for the spectrophotometric assay. 3 mL of working $ABTS^{\bullet+}$ solution were mixed with 10 µL of sample (concentration $1x10^{-3}$ mol/L) and the absorbance value was measured after a reaction time of 30 minutes at of λ=734 nm wavelength corresponding to the maximum of the absorbance. Results were expressed as trolox equivalents. Three replicate samples were analysed in each case.

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