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Development of Eco-friendly Microextraction-TLC Approach for Diphenhydramine Detection in Different Samples

MAHAK MALVIYA, SAKSHI PANDEY and LALIT P. CHANDRAVANSHI*

Department of Forensic Science, School of Allied Health Sciences, Sharda University, Greater Noida-201310, India

*Corresponding author: E-mail: chandravanshi04@gmail.com

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Diphenhydramine (DPH) is an antihistamine drug commonly incorporated into pharmaceutical formulations such as cough syrups, antipyretics and anti-allergy medications. It has been a widely abused drug in over-the-counter medicines for day-to-day use without any prescription. Therefore, a new, greener, rapid and cost-effective methodology was developed by integrating vortex-assisted dispersive liquid-liquid microextraction (VA-DLLME) with TLC coupled to image colorimetry for the detection of diphenhydramine in biological and pharmaceutical samples. Optimisation of DPH was performed by evaluating extraction solvent and its volume (trichloroethylene, 100 µL), disperser solvent and its volume (methanol, 1000 µL), pH (11) and vortex speed and time (2000 rpm, 1 min). The sediment phase obtained from the VA-DLLME method was subjected to TLC-image colorimetry, where ethyl acetate: ammonia solution: methanol (42.5:2.5:5 v/v/v) was used as the mobile phase. The developed TLC plate was photographed under 254 nm UV light and the image was analysed using ImageJ software. Further, validation was performed according to ICH guidelines assessing parameters including the limit of detection (2.08, 1.56, 1.56 µg/spot), the limit of quantification (6.25, 3.12, 5.61 µg/spot), linearity, accuracy, precision (inter-day and intra-day), recovery (119.70%, 141.99%, 172.53%) and enrichment factor (94.74%, 90.64%, 80.47%) for blood, urine and pharmaceutical formulation, respectively.

Keywords: Diphenhydramine hydrochloride, DLLME, Thin-layer chromatography, Image analysis, Greenness/whiteness.

INTRODUCTION

Diphenhydramine hydrochloride (DPH·HCl), chemically known as 2-benzhydryloxyethyldimethylamine hydrochloride ($C_{17}H_{21}NO$·HCl), is a commonly used drug that appears as an almost white, odourless compound with a bitter taste [1]. It is a first-generation histamine agonist that acts primarily as an anti-allergic drug [2]. As it is an antihistamine drug has anti-cholinergic and sedative effects therefore, when administered can cause lethargy or sleepiness [3-5].

DPH, widely available over-the-counter drug, commonly used to manage the symptoms of fever, common cold, allergic rhinitis and to induce sleep in insomniac patients [6-8]. DPH acts as an antagonist for the H1 histamine receptor, suppressing capillary inflammation and reducing symptoms of allergic reactions. It competes with the H1 receptor sites of the central nervous system that are responsible for sedating side effects [9,10]. The anticholinergic effect is due to its action upon muscarinic receptors, leading to blurry vision, dry mouth and tachycardia as the increase in the concentration of DPH acts as a blocker for the heart's rectifier K^+ ion channel in the body [10]. However, it can cause various severe conditions like delirium/psychosis, seizure, cardiac arrhythmia and coma [10,11].

Although DPH, is considered a non-fatal drug it can be a cause of drug-dependent toxicity [12]. The consumption of single-use DPH medication has been known to cause homicidal death in children is 60 %, abuse as a sleeping aid is 40 % and unsupervised accidental ingestion is 83% of the cases between the years 2-10 years. The bimodal distribution of DPH has been related to the trends of abuse, misuse and suicide cases among kids (age 10-14 years) and older adults (55+ years) [13]. However, the intentional consumption of DPH was found to be 86%, of which the cause was self-harm and 11.1% was unintentional/accidental in adults [14]. Drug extraction has been performed for a long time; the two traditional methods are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). LLE is a solvent-based extraction technique where the

two immiscible phases (aqueous phase and organic phase) are poured into a separating funnel to extract the desired analyte [15,16]. Whereas, SPE is the sorbent-based extraction technique that uses the adsorption (solid phase and aqueous phase) of the desired analyte onto a sorbent and then its extraction from the sorbent [15,16]. Although LLE and SPE have various advantages, they are expensive, time-consuming and environmentally unfriendly, use large amounts of solvents, are laborious, hazardous to human health and unhandy [17].

Microextraction, a modern miniaturized sample preparation technique requiring little or no organic solvent, has gained significant attention among researchers. These methods are rapid, eco-friendly, cost-effective and highly efficient, with compatibility across various analytical instruments and widely applied in food, pharmaceutical, chemical and forensic sciences, and is broadly classified into sorbent-based and solvent-based microextraction technique [17]. In this study, dispersive liquid-liquid microextraction (DLLME) technique was used, a solvent based microextraction technique, which has shown promising results over LLE and SPE [18]. It is affordable, user-friendly and less time-consuming, has high sample throughput and is eco-friendly [19]. Classical DLLME involves a third phase known as the disperser phase, which disperses the analyte into micelles (cloudy formation) that help to increase the surface area for the analyte to be extracted in the aqueous phase [20,21]. Upon centrifugation, this cloudy formation settles down in a sediment phase, which is used for further analysis. Similarly, TLC has long been used for detecting drugs and their metabolites due to its simplicity, low cost, minimal solvent use, rapid analysis and high sample throughput [20]. When combined with image-based analysis using software such as ImageJ, TLC becomes a rapid, reliable and cost-effective approach for qualitative analysis, as images of analyte spots can be converted into numerical data for evaluation [21,22]. The portability and ability of this method is to deliver immediate results have increased its relevance in analytical research.

No green, rapid and cost-effective method has been developed for detecting DPH in blood, urine and pharmaceutical formulations. This lack of availability has led to the development of the vortex-assisted dispersive liquid-liquid microextraction thin-layer chromatography image analysis (VA-DLLME-TLC image colorimetry) methodology. In this study, the developed method was optimized, validated and applied to the analysis of blood, urine and pharmaceutical formulation samples and its performance was compared with HPLC. The environmental sustainability and practical applicability of the method were evaluated using the Analytical GREEnness (AGREE) calculator and the Blue Applicability Grade Index (BAGI) metric, and the results were compared with previously reported analytical approaches [23,24].

EXPERIMENTAL

All the reagents and chemicals used in the study were of analytical grade until stated otherwise. The analytical grade standard of diphenhydramine hydrochloride (DPH·HCl, CAS no. 147-24-0, purity >98%) was obtained from Sigma-Aldrich, USA. Chlorobenzene (CB, purity >99%), carbon tetrachloride (CCl_4, purity >99%), chloroform (CF, purity >99%), dichloromethane (DCM, purity >99%) and trichloromethane (TCM, purity >99%) were used as extraction solvents obtained from SRL chemicals. Acetone (ACT, purity >99%), acetonitrile (ACN, purity >99%), ethanol (purity >99%) and methanol (purity >99%) were obtained from SRL chemicals and used as dispersive solvents. Pre-coated TLC aluminium plates (20 cm × 20 cm) with silica gel 60 F_{254} (cat no. 38221990) were procured from the Merck (Darmstadt, Germany). Ultrapure water for microextraction and HPLC-grade water were used for the study.

Stock solution preparation: The stock solution of DPH (10 mg/mL) was prepared in HPLC-grade water and diluted to prepare the working standard solution for optimisation and validation studies. The stock solution was stored at 4 ºC, which was used for optimizing and fortifying the biological sample for validation.

Samples preparation

Biological sample: The real blood and urine samples (self) were donated by the authors of this study (aged between 28-40 years) and stored at 4 ºC. A 0.5 mL of blank blood sample was taken into the 15 mL falcon tube and spiked with 50 µL of 10 mg/mL DPH stock solution followed by the addition of 1 mL of methanol/acetonitrile. This mixture was shaken well to precipitate the blood protein and centrifuge the mixture for 5 min at 5000 rpm. The separated serum was transferred into another tube for analysis using vortex-assisted dispersive liquid-liquid microextraction coupled with TLC image colorimetry (VA-DLLME-TLC image analysis) and HPLC. For urine analysis, 0.5 mL of blank urine was diluted with 5 mL of HPLC-grade water and spiked with 50 µL of pre-prepared DPH stock solution. The mixture was vortexed for 3-5 min and then used for VA-DLLME-TLC image colorimetry and HPLC analysis.

Pharmaceutical formulation sample: Vicks Action 500 were bought from the local pharmacy store and then 10 tablets were crushed into fine powder and the quantity average to the weight of 1 tablet was used. A 2.5 mL of HPLC-grade water was added to the sample and vortexed. Then this mixture was ultrasonicated for 10 min at room temperature and centrifuged at 5000 rpm for 5 min, the supernatant was collected in 2 mL tube. This prepared solution of DPH was used for VA-DLLME-TLC image colorimetry and HPLC analysis.

Vortex-assisted dispersive liquid-liquid microextraction (VA-DLLME): For blood sample, 0.5 mL of deproteinized blood was transferred into a Falcon tube and diluted with HPLC-grade water to a final volume of 5 mL. The pH 11 was maintained by adding 0.1 M solution of NaOH to the sample. This sample solution was subjected to VA-DLLME, by rapidly injecting the mixture with 100 µL TCM (extraction solvent) and 1000 µL MeOH (disperser solvent), which on amalgamation formed a cloudy solution. Then it was vortexed for 1 min and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and approximately 80 µL of sedimented phase was recovered and further used for TLC analysis. Similarly, the same methodology was adapted for urine and pharmaceutical formulation samples.

TLC image analysis: TLC analysis of the sedimented phase was done using pre-coated silica gel plates. The extract (5 µL) obtained by VA-DLLME procedure from blood, urine

and pharmaceutical formulation samples were spotted onto the plates by using micropipette 1 cm above the edge and left to air dry for 5 min at room temperature. The mobile phase was prepared suitable for DPH, composed of ethyl acetate-methanol-ammonia solution (42.5:5:2.5 v/v/v). The dried plate was placed in the development chamber (vertical development chamber), pre-saturated for 15 min with vapours of the mobile phase. The plate was developed to 12 cm from the starting point in ascending mode and was removed from the development chamber. Once air dried, this plate was observed under the UV-chamber and take a clear photograph of the plate with the help of a digital camera. The image was analysed with freely available ImageJ soft-ware and an image densitogram was obtained (Fig. 1). The formation of a densitometry chromatogram from a TLC plate using a digital camera and ImageJ software is based on the principle of optical density measurement through image-based densitometry. This approach estimated the relative optical density of analyte zones by analyzing the intensity of light reflectance captured in a digital image. Since optical density was directly proportional to analyte concentration, darker regions on the TLC plate corresponded to higher analyte levels, forming the basis for generating a densitometric chromatogram. After developing the TLC plate, a high-resolution image was acquired under uniform illumination using UV light at 254 nm. The image was then uploaded into ImageJ and split into RGB (red, green, and blue) colour channels. Each channel was examined individually and the green channel provided the highest contrast, so it was selected for further analysis. Within the green channel, the optical density of each spot was represented by pixel-intensity values, where higher pixel density correlated with higher analyte concentration. Using the line-profile tool in ImageJ, a scan was performed along the TLC lane to generate a densitometric chromatogram. In this chromatogram, the X-axis represented migration distance (R_f range), while the Y-axis displayed pixel-intensity values, indicating optical density.

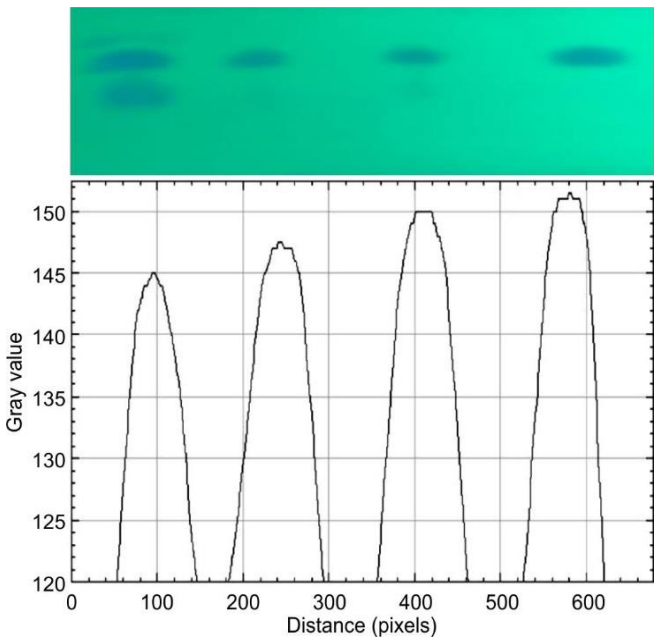


Fig. 1. Image and densitogram of diphenhydramine in pharmaceutical, urine, blood and standard samples

HPLC analysis: HPLC, (Waters, Breeze QS HPLC, USA) was used for the identification of DPH in blood, urine and pharmaceutical formulations. The instrument was calibrated with the DPH-HCl standard (Sigma-Aldrich, USA) and the linear plot was obtained (10 ppm to 100 ppm). The mobile phase was selected as ammonium acetate buffer (pH: 6.8, 15 mM) and acetonitrile (60:40 v/v), which was filtered with 0.45 μm filter paper and vortex agitated for 15 min. The flow rate of the mobile phase was maintained at 1 mL/min at isocratic mode. The sediment phases of each sample were diluted to make up to 1 mL by adding HPLC-grade water. The 20 μL of each sample was injected into the column (reverse phase, C_{18}) and the run-time was standardised at 25 min. The chromatograms are shown in Fig. 2.

Analysis of DPH·HCl was carried out on a Water RP-HPLC system module with Photodiode array detector and reversed-phase C_{18} column (Atlantis dC18 5 μm, 4.6 mm × 150 mm) and the wavelength of detection was kept between 200-400 nm. Empower QS software was used to analyze the HPLC chromatographic data.

RESULTS AND DISCUSSION

Screening of TLC parameters: Two most suitable solvent systems screened from the literature for the analysis of DPH *viz.* chloroform:methanol (90:10 v/v) and ethyl acetate:ammonia solution:methanol (42.5:2.5:5 v/v/v). The mobile phase was optimized through a series of trials and a solvent system consisting of ethyl acetate:ammonia:methanol (42.5: 2.5:5, v/v/v) provided effective separation of DPH. Chamber saturation time was also evaluated between 15 and 30 min, as separation visibility was influenced by this parameter. A 15 min saturation time produced the most distinct visualization of DPH under UV illumination. As a result, ethyl acetate: ammonia:methanol (42.5:2.5:5 v/v/v) (Fig. 3) was selected as a solvent system, with a 15 min saturation period for DPH.

Screening of extraction solvents: The process of DLLME requires extraction solvents which is denser than water, therefore in this procedure 5 solvents namely, DCM (d = 1.32), CF (d = 1.48), CCl_4 (d = 1.59), CB (d = 1.11) and TCM (d = 1.46) were used as extraction solvents. The most suitable extraction solvent among the 5 solvents was chosen by performing a series of experiments. Each extraction solvents of volume 100 μL and constant disperser solvent (EtOH) of 500 μL fixed volume were mixed and rapidly injected into the aqueous containing 15 μL of DPH, resulting in the formation of a cloudy solution. Then the mixtures were vortex-agitated for 1 min and centrifuged for 3-5 min at 5000 rpm. The supernatant was discarded and the sediment phase, approximately 80 μL was collected, out of which 5 μL was spotted on the TLC plate. Once developed, these spots were observed under the UV light chamber, which were photographed and processed through ImageJ software. A densitogram was plotted, displaying the quantity of each of the five spots on the TLC plate. Fig. 4a shows that TCM has the highest extraction efficiency as the extraction solvent for DPH. Similarly, in further experiments, the extraction solvent (TCM) volume was optimised by taking three volumes of TCM ranging between 100-125 μL and fixed disperser solvent EtOH at 500 μL volume. The rest of the

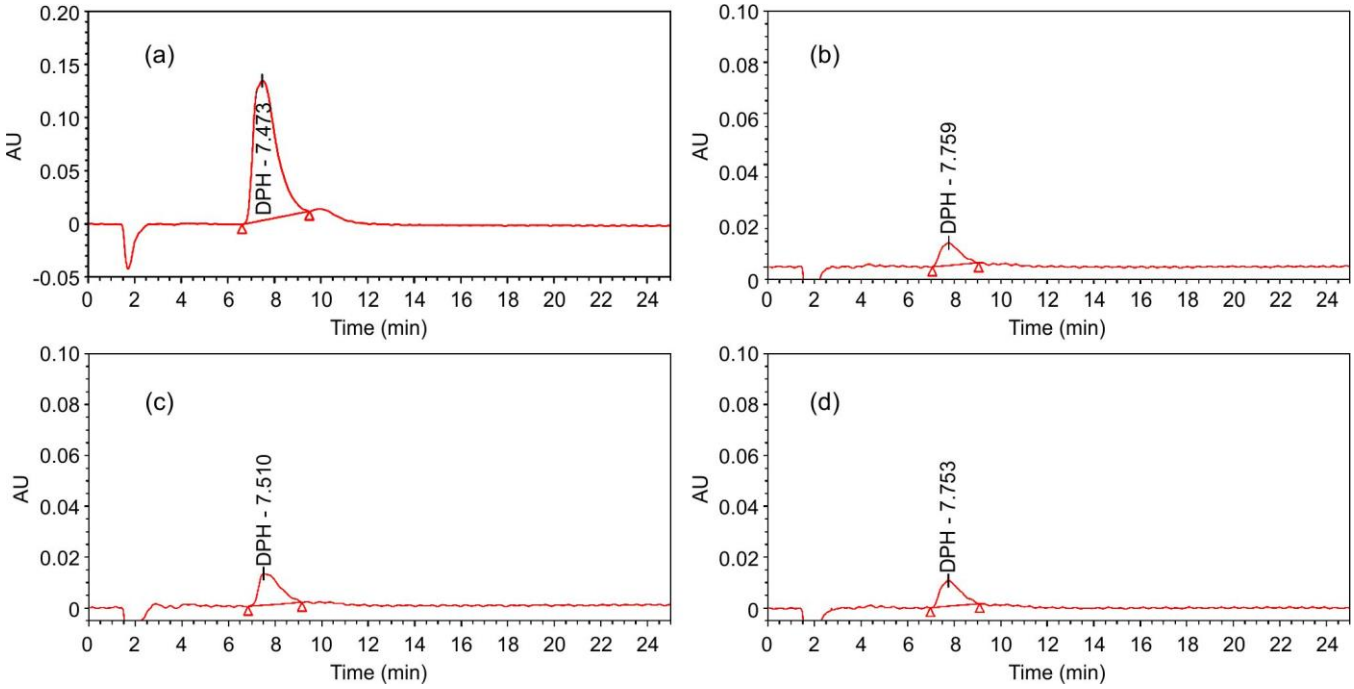


Fig. 2. Chromatograms of (a) standard diphenhydramine, (b) blood sample, (c) urine sample and (d) pharmaceutical formulation

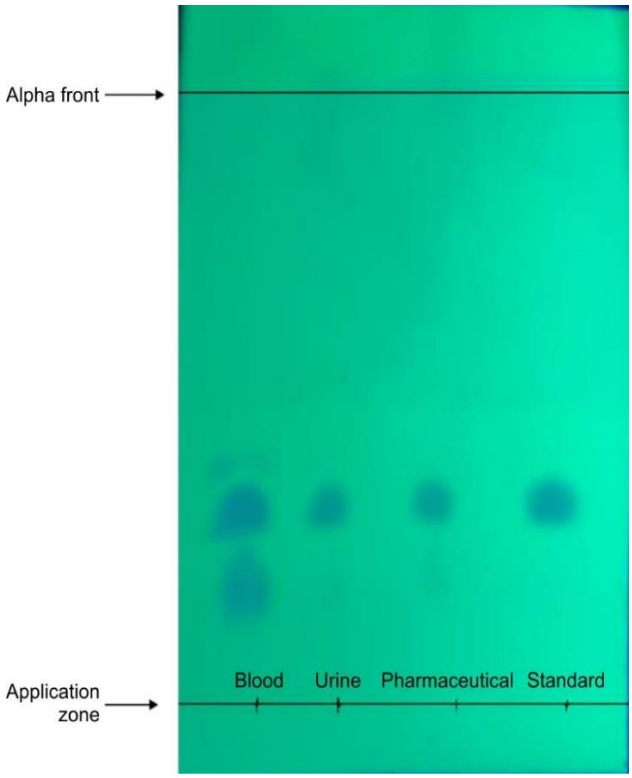


Fig. 3. Alpha front image of developed TLC plate

process for VA-DLLME was the same as mentioned above. Fig. 4b shows that DPH exhibited the highest extraction efficiency when TCM was used at a volume of 100 µL.

Screening of disperser solvents: The screening of 5 disperser solvents was done to select the most suitable for DPH among ACT, ACN, EtOH and MeOH. Disperser solvents are rapidly injected into the solvent system to form a cloudy solution. These tiny droplets help increase the surface area for the analyte to achieve higher extraction and equilibrium. The four disperser solvents (500 µL) were added to the previously selected extraction solvent TCM (100 µL). This mixture was rapidly injected into the aqueous sample of DPH. These mixtures were vortex-agitated for 1 min and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the sediment phase was spotted on the TLC plate. Fig. 5a shows that MeOH has the highest peak area for DPH when used as a disperser solvent. Similarly, the different volumes between 500-1200 µL of MeOH (disperser solvent) were rapidly injected into the aqueous solution of DPH with 100 µL of TCM (extraction solvent). After vortex-agitating for 1 min and centrifuging for 5 min at 5000 rpm, the sediment phase was spotted on the TLC plate. The volume of 1000 µL of MeOH in Fig. 5b indicated the highest peak area for DPH, therefore selected as volume for the disperser solvent for further experiments.

Screening of pH and ionic strength: In DLLME, pH is a critical factor influencing analyte extraction, as efficient partitioning into the organic phase requires the analyte to remain predominantly in its molecular (non-ionized) form in the aqueous medium. The molecular state of the analyte is determined by its pK_a value. According to the Henderson-Hasselbalch equation, acidic drugs are approximately 99% ionized at two pH units above their pK_a and about 1% ionized at two pH units below their pK_a. Based on this principle, the effect of pH on the extraction of DPH (pK_a = 8.98) was investigated over the pH range of 7-12. The aqueous-phase pH was adjusted using 0.1 M NaOH and 0.1 M HCl, while keeping others optimized parameters constant. As shown in Fig. 6a, the highest DLLME extraction efficiency for DPH was obtained at pH 11. The influence of ionic strength was also evaluated by adding NaCl (0-10%, w/v) to the aqueous

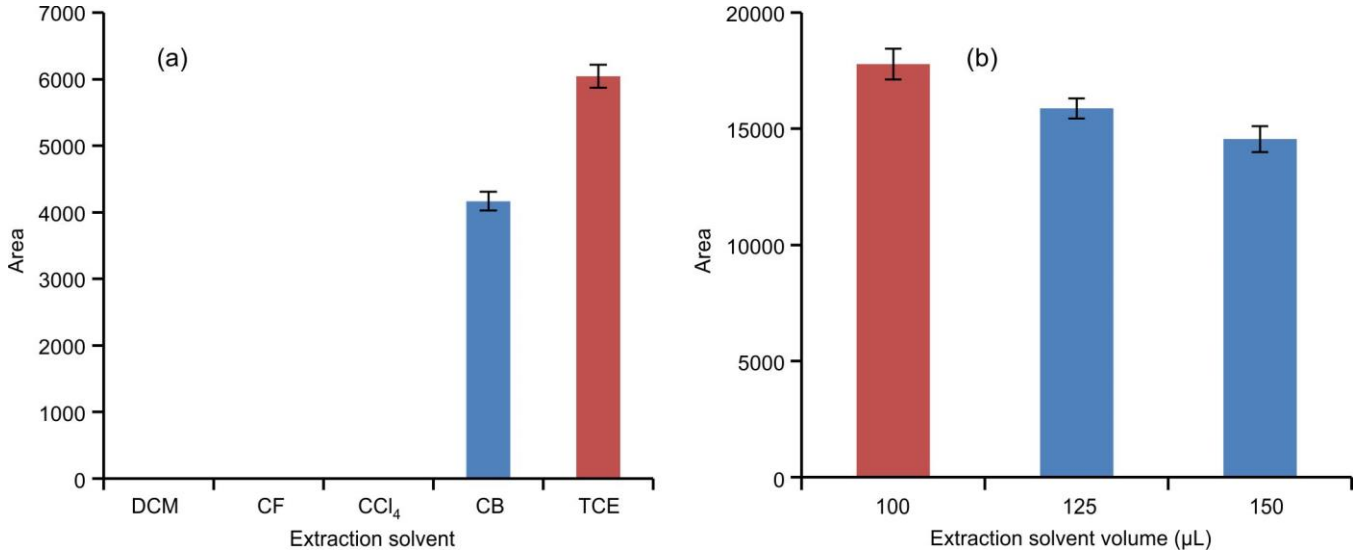


Fig. 4. Screening of (a) extraction solvent and (b) its volume

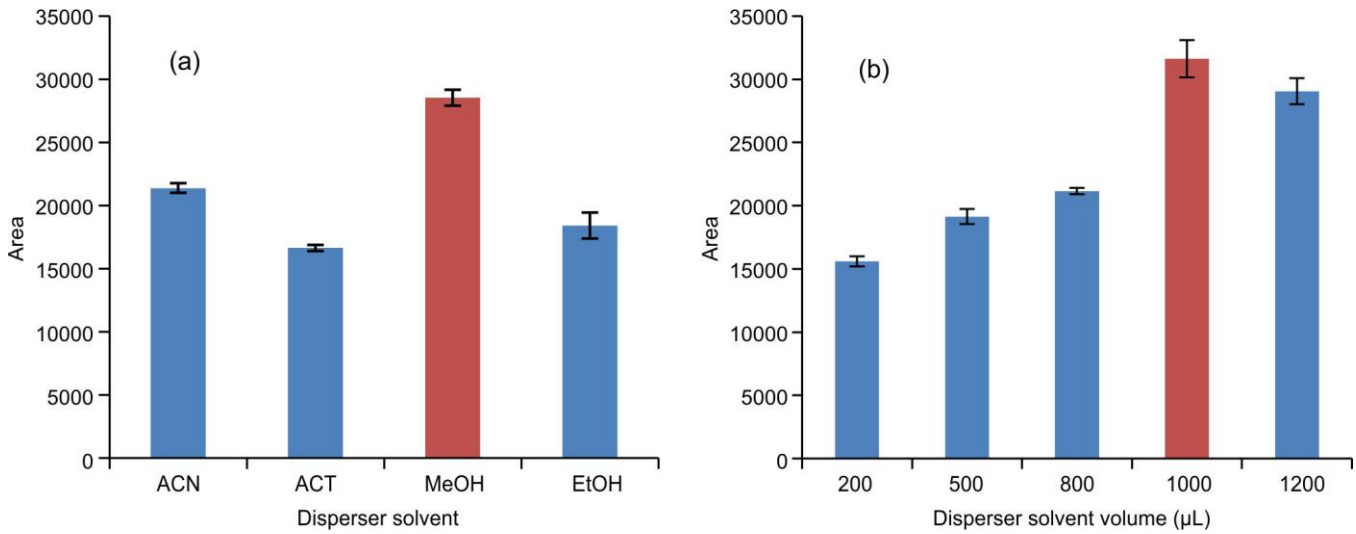


Fig. 5. Screening of (a) disperser solvent and (b) its volume

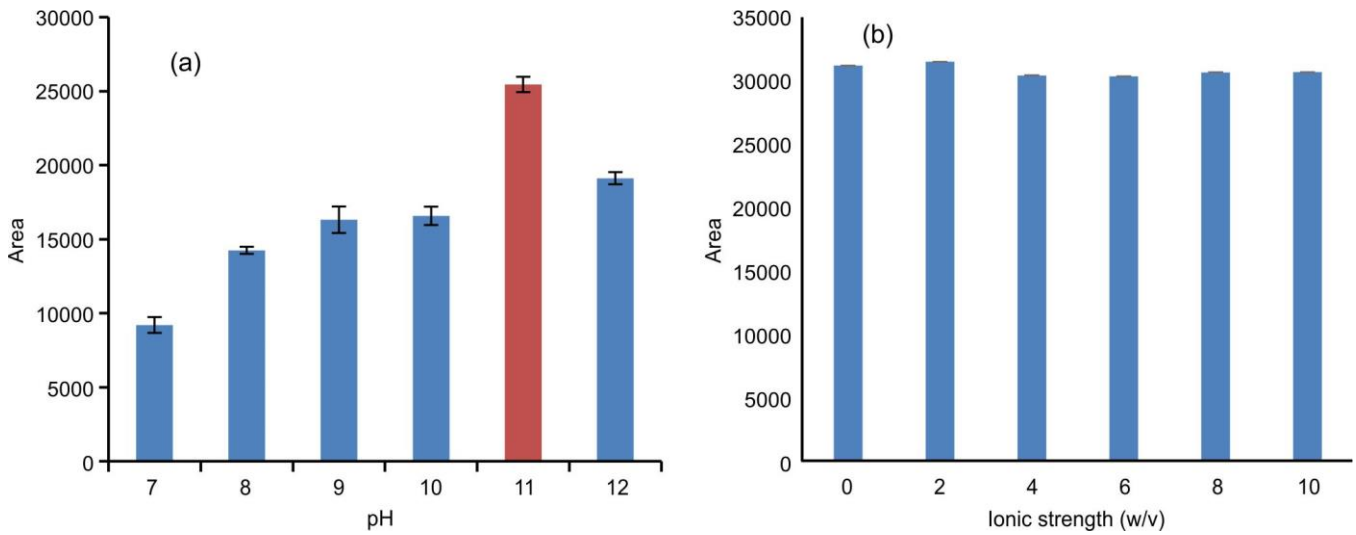


Fig. 6. (a) Screening of pH and (b) ionic strength

TABLE-1
LIMIT OF DETECTION, LIMIT OF QUANTIFICATION, LINEARITY AND R^2 VALUE OF BLOOD, URINE AND PHARMACEUTICAL FORMULATION

Samples	LOD		LOQ		Regression equation	R^2
Blood	2.08 µg/spot	0.130	6.25 µg/spot	0.840	y = 3930.8x	0.991
Urine	1.56 µg/spot	0.165	3.12 µg/spot	0.616	y = 3570.8x	0.997
Pharmaceutical formulation	1.56 µg/spot	0.370	5.61 µg/spot	1.120	y = 380.31x	0.995

phase to reduce analyte solubility and potentially enhance extraction efficiency. However, no significant improvement in DPH extraction was observed, and therefore salt addition was omitted in subsequent DLLME experiments (Fig. 6b).

Screening of vortex agitation speed and time: Vortex agitation was performed to understand its effect on extraction efficiency. It provided emulsification of the extraction solvent in the aqueous phase, which improves the interfacial area for the mass transfer of analyte by enhancing the extraction solvent's rate by turning it into microdroplets. The partition equilibrium of the analyte is also attained in a few minutes as vortex agitation reduces the diffusion distances. The vortex agitation was performed between 0-4000 rpm for 1 min, keeping all the other parameters as optimised. Fig. 7a shows the maximum extraction was achieved at 2000 rpm and decreased beyond this speed. Similarly, the effect of duration on vortex agitation was studied between 1-5 min (1, 3 and 5 min), while Fig. 7b shows that vortex agitation produced maximum extraction of DPH at 1 min, after which the extraction efficiency gradually declined.

Validation: The entire methodology was validated according to the International Council of Harmonisation (ICH) guidelines. The limit of detection (LOD) and the limit of quantification (LOQ) (Table-1) were calculated according to the standard curve as obtained by using the following formula:

$$LOD = \frac{3.3\sigma}{S}; \qquad LOQ = \frac{10\sigma}{S}$$

where σ denotes the standard deviation of the response and S denotes the slope of the calibration curve. The linearity (Table-1) was plotted between the concentrations of 6.25-38.88 µg/spot in blood (Fig. 8a), 3.12-44.44 µg/spot in urine (Fig. 8b) and 1.56-43.75 µg/spot in pharmaceutical formulation samples (Fig. 8c) (n = 3). Also, the accuracy, relative recovery (RR%) and enrichment factor (EF) were evaluated (Table-3). The inter-day and intra-day precision (Table-2) were also calculated (Table-3).

TABLE-2
ENRICHMENT FACTOR AND PRECISION DATA

Samples	EF%	Conc. (µg/spot)	Precision	
			Intra-day (%RSD)	Inter-day (%RSD)
Blood	60.7	6.25	5.8	7.2
		16.66	6.3	7.9
		38.88	5.5	8.2
Urine	68.4	3.12	3.2	5.6
		22.22	4.5	4.2
		44.44	4.1	5.1
Pharmaceutical formulation	80.5	1.56	1.5	3.4
		14.28	2.2	3.9
		43.75	1.8	2.7

Application to real samples and analysis: The present method (VA-DLLME-TLC image colorimetry) was successfully applied to quantify DPH in human blood, urine and pharmaceutical formulations under optimised and validated conditions. Participants aged 28-40 years who were under medication of Vicks Action 500, donated their blood and urine samples after the regular treatment for two days. Blood and urine samples were received from the patients after 6 h of the last dose taken and kept the sample at ~4 ºC until anal-

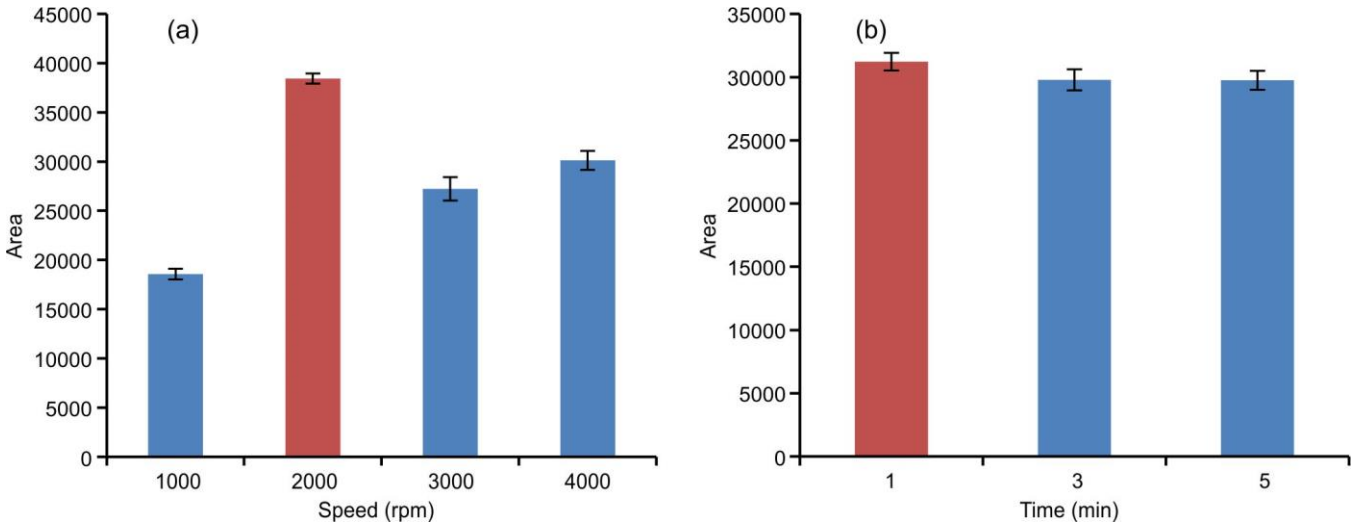


Fig. 7. Screening of (a) vortex speed and (b) time

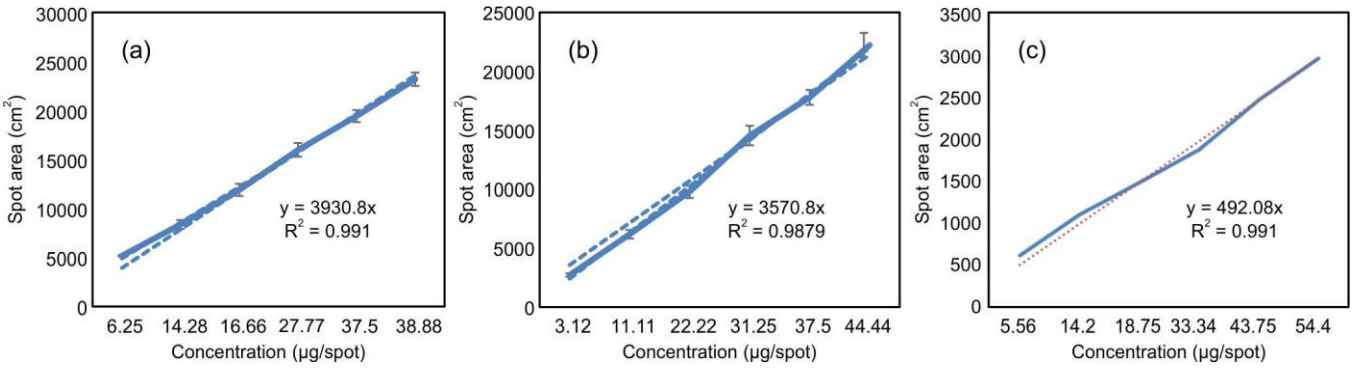


Fig. 8. Graphical representation of linearity in (a) blood, (b) urine and (c) pharmaceutical formulation samples

TABLE-3
ACCURACY AND RELATIVE RECOVERY DATA

Samples	Conc. (µg/spot)	Accuracy (%) (µg/spot)	RR (%) (µg/spot)
Blood	6.25	90.3	78.4
	16.66	95.1	73.2
	38.88	93.4	70.2
Urine	3.12	98.7	86.3
	22.22	97.1	88.2
	44.44	95.2	84.3
Pharmaceutical formulation	1.56	99.8	92.1
	14.28	103.4	95.0
	43.75	101.2	96.2

ysis. Similarly, the sediment phase from VA-DLLME-TLC image colorimetry was subjected to HPLC analysis, for which the retention times for DPH were observed at 7.759, 7.510 and 7.753 for the blood, urine and pharmaceutical formulation samples, respectively. It was found that the results for both VA-DLLME-TLC image colorimetry and HPLC analysis were in close agreement. The levels of DPH in blood, urine and pharmaceutical formulation samples as determined by VA-DLLME-TLC image colorimetry and HPLC analysis are shown in the Tables 4 and 5, respectively.

TABLE-4
DETERMINATION OF HUMAN BLOOD AND URINE SAMPLES COLLECTED AFTER 6 h OF ADMINISTRATION OF DIPHENHYDRAMINE (500 mg) (n = 3)

Sample	TLC-image analysis (µg/spot) (%RSD)	HPLC analysis (µg/mL) (%RSD)	Agreement of TLC image processing with HPLC analysis (%)
Blood	128.69 (3.46)	129.82 (4.94)	99.12
Urine	427.98 (1.89)	412.97 (2.44)	103.63

Greenness characteristics of the proposed method: The greenness and practicability calculations were performed by analytical greenness (AGree) and blue applicability grade index (BAGI), which is based on the green analytical chemistry (GAC) and 'blue' principle of white analytical chemistry (WAC). The AGree calculator depicts the score in the form of a pictogram, which is circular. The circle's center denotes the actual score between 0-1, which indicates 0 being the least and 1 being the greenest methodology. The outer circle weights the 12 principles of GAC according to their yield, which can be manually adjusted from 1 to 4. Moreover, the colour range from green to red helps in understanding the greenness of the methodology.

BAGI stands for Blue Applicability Grade Index is a metric tool developed by Manousi *et al.* [21] and is based on the 'blue' principle of white analytical chemistry (WAC) proposed by Nowak *et al.* [22]. This metric tool focuses on the applicability of the developed method in terms of operational simplicity, cost-effectiveness and low time consumption. The tool creates an asteroid pictogram in which the value is indicated at the centre from 25 to 100, where 25 means the least and 100 means the most applicable. Along with this, the pictogram also depicts the different shades varying from white to light blue to medium blue to navy blue, where white shows the least applicability and navy blue shows the most applicability. The score in the centre should be above 60 for a method to be considered applicable, which is created by the set of 10 questions, including analysis type, multiple or single analysis, analytical technique, simultaneous sample preparation, samples per hour, reagents/materials, degree of automation and amount of sample. The user can choose the sub-categories in the mentioned headlines to create the pictogram in accordance with the developed methodology. A comparative study of the proposed methodology is performed by calculating the greenness and applicable using AGree and BAGI metric tools.

TABLE-5
DETERMINATION OF DIPHENHYDRAMINE IN PHARMACEUTICAL FORMULATIONS (n = 3)

Sample (claimed DPH)	Concentration prepared (µg/mL)	Concentration found		Amount of DPH found (mg)		Agreement of TLC image processing with HPLC analysis (%)
		TLC image processing (µg/spot)	HPLC analysis (µg/mL)	TLC image processing (mg)	HPLC analysis (mg)	
Pharmaceutical formulation (500 mg)	600	517.29	503.61	431.0	419.5	97.35

TABLE-6
TABULAR REPRESENTATION OF THE COMPARATIVE RESULTS OF THE AGree CALCULATOR AND BAGI METRIC TOOLS OF THE VA-DLLME-TLC-IC WITH PREVIOUS STUDIES ON DIPHENHYDRAMINE

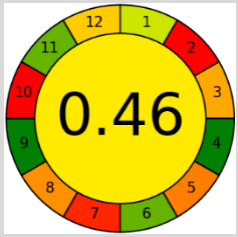
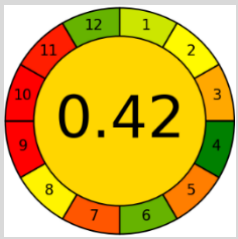
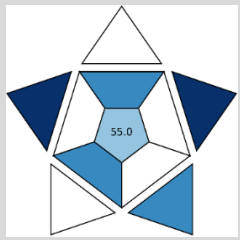
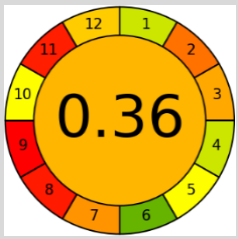
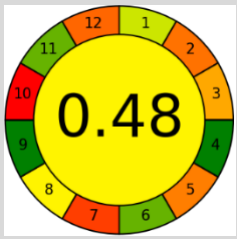
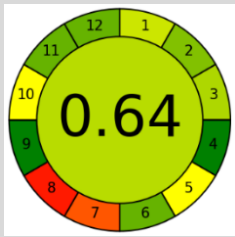
Sample matrix	Sample pre-treatment and extraction method	Technique	AGREE score	BAGI score	Ref.
Pharmaceutical formulation	No extraction	HPTLC			[1]
Pharmaceutical formulation	Solid phase extraction	SPE-HPLC			[23]
Pharmaceutical formulation	No extraction	RP-HPLC			[24]
Pharmaceutical formulation	No extraction	SFS			[25]
Blood, urine and pharmaceutical formulation	Dispersive liquid-liquid microextraction	VA-DLLME-TLC-IC			Present study

Table-6 summarizes the scores obtained by previously reported methods for the detection of DPH in comparison with the VA-DLLME-TLC-DIC approach. The results indicate that the proposed methodology achieves a higher score in meeting the principles of Green Analytical Chemistry (GAC) and White Analytical Chemistry (WAC).

Conclusion

A vortex-assisted dispersive liquid-liquid microextraction coupled with TLC image colorimetry (VA-DLLME-TLC-DIC) method was successfully developed and validated for the determination of diphenhydramine (DPH) in biological and pharmaceutical samples. The optimized chromatographic and extraction parameters including mobile phase composition, extraction solvent, disperser solvent, pH and vortex conditions, resulted in efficient extraction and reliable separation of DPH. The method demonstrated satisfactory linearity, precision, accuracy, recovery and enrichment performance in blood, urine and pharmaceutical formulation samples. Application to the real samples confirmed that the proposed approach produced results comparable to those obtained by HPLC analysis, supporting its analytical reliability. The method requires minimal solvent consumption, simple instrumentation, and short analysis time, making it suitable for routine laboratory

use. Evaluation using AGREE and BAGI metrics further indicated favorable environmental sustainability and practical applicability compared with previously reported methods. Thus, the proposed method has immense scope in resource-limited laboratories and is simple to perform. It also does not require overnight preparatory steps, which can be used for the detection of highly sensitive analytes as well. Apart from its use in resource-limited laboratories, this methodology is also compatible with hyphenated techniques like HPLC, GC-MS, LC-MS, *etc*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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