

## Automated Quantification of Doxylamine and Diphenhydramine in Human Plasma using on-line extraction-HPLC-DAD (TOX.I.S.)

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**Key words:** Intoxication, doxylamine, diphenhydramine, automated quantification, plasma, HPLC-DAD (TOX.I.S.)

### Abstract

**Aim:** The purpose of this study was to develop a screening method for the identification of weak basic, neutral and weak acidic substances in human plasma using on-line extraction-HPLC-DAD (TOX.I.S. - toxicological identification system). Beside the identification of xenobiotics, the method was validated for the automatic quantification of two compounds in human plasma: doxylamine (DA) and diphenhydramine (DPH).

In the context of STA routine analysis more than 450 intoxication cases were analyzed by the described method. Two intoxication cases are presented.

**Methods:** The quantification of DA and DPH in human plasma was based on a methanolic extraction of plasma (0.2 ml) and basic (pH = 9) automated on-line extraction (Strata-X, 25 µm, 20x2 mm) followed by on-line extraction followed by HPLC-DAD detection (TOX.I.S.). Analytical separation was carried out on Gemini NX (150x4.6 mm, 3µm) using gradient elution. The mobile phase consisted of 0.05M potassium dihydrogen phosphate buffer (pH=2.3) and acetonitrile/water (90/10, v/v). Peak identification, retention time (RT) and relative retention time (RRT) were carried out by chromatographic and spectral data comparison with a library containing >750 UV-spectra of weak basic, weak acidic and neutral compounds. Criteria for peak identification were a 95% agreement between the measured and the library spectrum (similarity  $\geq 0.995$ ) and a maximum difference in RRTs of  $\pm 5\%$ . The validation procedure was performed according to the GTFCh guidelines.

**Results and Discussion:** A new method for the identification of xenobiotics in human plasma was successfully developed, integrated and validated using TOX.I.S. The tested model compounds for the automated quantification yielded following results: the calibration range of DA and DPH was linear from 0.25-5.0 mg/L ( $r^2 = 0.999$ ). The LLOQs (0.15 mg/L for DPH and 0.25 mg/L for DA) were low enough for the detection of intoxications with these substances. In intoxication **case 1**, DPH was detected in toxic concentration (3.24 mg/L); Dinordiphenhydramine and nordiphenhydramine were determined qualitatively. In **case 2**, sub-therapeutic concentrations of DPH (0.29 mg/L) and of DA (<0.25 mg/L) were found. Moreover, amitriptyline (0.084 mg/L) and nortriptyline (0.158 mg/L) and other metabolites of amitriptyline were quantitated in the same analytical run. Concentrations of amitriptyline and of nortriptyline were in therapeutic range.

**Conclusion:** In TOX.I.S., an additional method for the automated identification of a broad range of weak basic, weak acidic and neutral compounds in human plasma was integrated and successfully validated. A highly automated online-SPE-HPLC-DAD method was capable of identifying (and automated quantification of doxylamine and diphenhydramine) xenobiotics in human plasma including sample preparation, data evaluation and comprehensive reporting.

## 1. Introduction

The fully automated, qualitative screening method (TOX.I.S.: on-line extraction-HPLC-DAD) for the identification of basic compounds in urine is used in routine clinical toxicology use [1-2]. The purpose of this study was to develop and to integrate a further screening method for the identification of weak basic, neutral and weak acidic substances in human plasma using the same analytical system. Beside the identification of xenobiotics, the method was also validated for the automatic quantification of two model compounds in human plasma: doxylamine (DA) and diphenhydramine (DPH).

In the context of STA routine analysis more than 450 intoxication cases were analyzed by the described method.

With two application examples of poisoning cases we presented the automatic quantification of DA and DPH and the identification of weak basic, neutral and weak acidic compounds in the same human plasma samples using TOX.I.S.

Doxylamine (DA) and diphenhydramine (DPH) are members of the ethanolamine class of first generation competitive histamine H<sub>1</sub>-receptor antagonists and have anticholinergic, hypnotic, sedative, local anesthetic and antitussive properties (Fig. 1.) [3-4]. DA and DPH used for the treatment for sleep disorders and are often purposely taken in suicide attempts [5-6]. The effective daily dosage for DPH is between 30 and 100 mg and for DA [7] between 25 and 50 mg [8]. The lethal dose for DPH and DA is 40 mg/kg [9] and 25 - 250 mg/kg, respectively [10]. DA and DPH should not be combined with ethanol, cocaine, morphine, barbiturates, benzodiazepines or other antihistamines, because of synergistic effects [11-12]. The therapeutic range of DA and DPH in plasma is 0.1-0.6 mg/L and 0.2-1.0 mg/L [13]. The critical concentration in plasma for DA is specified as 3 mg/L and for DPH as 5 mg/L [13].

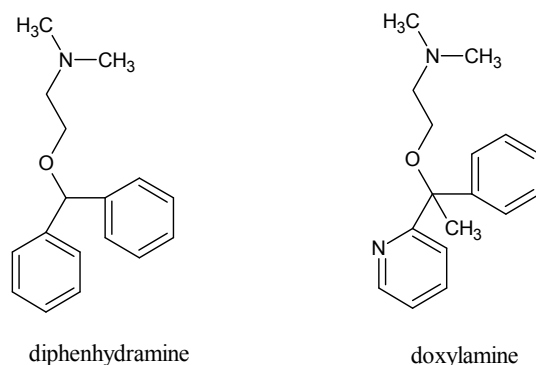


Fig. 1. Chemical structures of diphenhydramine and doxylamine.

Symptoms for DA and/or DPH poisonings are dry mouth, coordination disorders, inability to concentrate, restlessness, anxiety, states of agitation, hallucinations - in severe cases seizures, cardiac arrhythmias, ataxia, anticholinergic syndrome, psychosis and motoric unrest. Lethal poisonings are characterized by coma, heart / acute respiratory distress syndrome and major seizures [14].

DA and DPH undergo hepatic metabolism. The main metabolites of DPH are dinordiphenhydramine, nordiphenhydramine and diphenylmethoxyacetic acid, which conjugated to glutamine or glycine conjugates [15]. Plasma protein bindings are 85 to 99% for DPH [15] and 60 to 70% for DA [16]. The elimination half-life of DPH is 4 (range: 2.4 to 9.5 h) hours. Only 1% of DPH were eliminated unchanged in the urine [17]. Nordoxylamine, dinordoxylamine and

acetyl conjugates are main metabolites of DA [18]. The elimination half-life time of DA is 10 hours [16].

## Case histories

### Case 1

A 28-year old male (185 cm, 85 kg) was admitted to hospital with clinical symptoms of alcohol intoxication. His face and hands were reddened. Poisoning by drug intake was not ruled out.

### Case 2

A 41-year old woman (170 cm, 65 kg) was admitted to hospital in a confused mental state with involuntary movements. The woman was suffering from depression. A rapid urine test carried out in the hospital was positive for tricyclic antidepressants.

## 2. Material and Methods

### 2.1. Plasma samples

Plasma samples from intoxications were obtained from emergency rooms, hospitals and psychiatric institutes. The samples were stored at 7°C.

### 2.2. Sample preparation

300 µl of internal standard (*N*-ethyloxazepam,  $c = 1.0$  g/L) in methanol were added to 200 µl plasma sample. The mixture was vortexed for 5 min. by 2000 cycles per minute and centrifuged for 4 min. at 14600 x g. The supernatant (300 µl) was transferred to a new 1.5 ml plastic cup, diluted with 1.0 ml 0.1 M  $\text{NH}_4\text{HCO}_3$  buffer (pH = 9) and placed into the auto sampler. The injection volume was 1.0 ml.

### 2.3. Instrumentation

The TOX.I.S. consists of degasser (2x DGU-20A5), pumps (LC-20AT and LC-20 AB), high-pressure valve (FCV-20H2), column switching valves (2 x FCV-14AH and 2 x FCV-12AH), auto sampler (SIL-20AC), column oven (CTO-20AC), photodiode array detector (SPD-M20A) and a system controller (CBM-20AC), all from Shimadzu (Germany).

### 2.4. On-line extraction and chromatographic conditions

For on-line solid-phase extraction a 0.1 M  $\text{NH}_4\text{HCO}_3$  load buffer (pH = 9) was used. The compounds were eluted from Strata-X extraction column (20 x 2.0 mm, 25 µm, Phenomenex, Germany) onto a Gemini NX C18 column (150 x 4.6 mm, 3 µm, Phenomenex, Germany), with a precolumn of the same type (oven temperature: 40°C). The mobile phase consisted of ACN:H<sub>2</sub>O (90:10, v/v) and 0.05 M  $\text{KH}_2\text{PO}_4$  buffer (pH 2.3). The flow rate was 0.6 mL/min and total analysis time (including extraction) was 46 min.

### 2.5. Identification and quantification

The relative retention time (RRT) >95% and similarity >99% in agreement with 700 UV-spectra of weak basic, weak acidic and neutral compounds in in-house library were used to

identify the compounds in the chromatograms. Using LCSolution-software (Shimadzu, Germany) the concentration of compounds were calculated and reported automatically.

For calibration, drug-free, blank plasma samples were spiked with DA and DPH (0.25, 0.5, 0.75, 1.0, 2.5 and 5.0 mg/L). Each point on the calibration curve were averaged from  $n = 8$  measurements of spiked samples. Two controls (0.6 and 1.2 mg/L) were prepared analogous to spiked samples of calibration curve.

### 3. Results and Discussion

Beside the identification of basic compounds in urine, a further method for the identification of weak basic, neutral and weak acidic substances in human plasma was successfully developed and integrated in the TOX.I.S. (on-line extraction-HPLC-DAD).

The new method was successfully validated according to the GTFCh guidelines [19]: e.g. the LLOQ was 0.15 mg/L for DPH and 0.25 mg/L for DA. Linearity was obtained from 0.25-5.0 mg/L with coefficients of correlation better than 0.999 for DPH and DA. The concentrations of DA and DPH were calculated and reported automatically.

In **case 1**, the analysis of plasma samples via HS-GC revealed the absence of ethanol poisoning ( $c < 0.03$  g/L), contrary to initial hypothesis. Using the improved TOX.I.S., 3.24 mg/L of DPH in plasma were found. In addition, DPH's main metabolites dinordiphenhydramine and nordiphenhydramine and diphenhydramine-N-oxide, caffeine and hydrocortisone were identified (Figure 2). Another metabolite diphenylmethoxy acetic acid could not be extracted from plasma due to the alkaline extraction conditions.

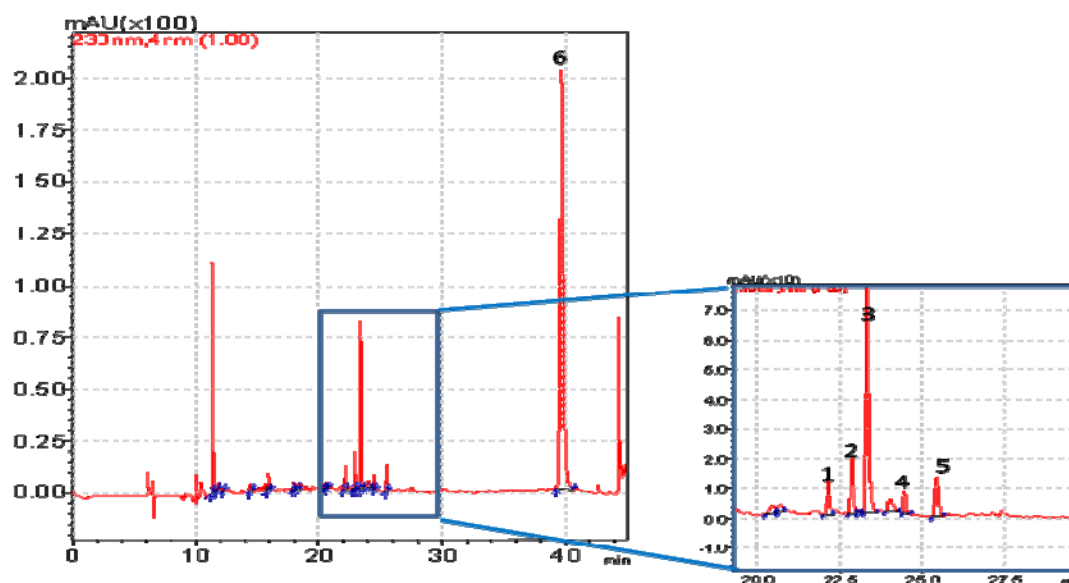


Fig. 2. Chromatogram of the plasma sample in case 1: 1 = dinordiphenhydramine, 2 = nordiphenhydramine, 3 = diphenhydramine (DPH), 4 = diphenhydramine-N-oxide, 5 = hydrocortisone and 6 = IS (N-ethyloxazepam).

In **case 2**, DA and DPH were identified and automatically quantified in the plasma sample (Figure 3). Concentrations were  $< 0.25$  mg/L (DA) and 0.29 mg/L (DPH) and were therefore in the lower therapeutic range. Nordoxylamine but also amitriptyline, nortriptyline and five further amitriptyline metabolites and hydrocortisone (Figure 3, Peak 9) were detected.

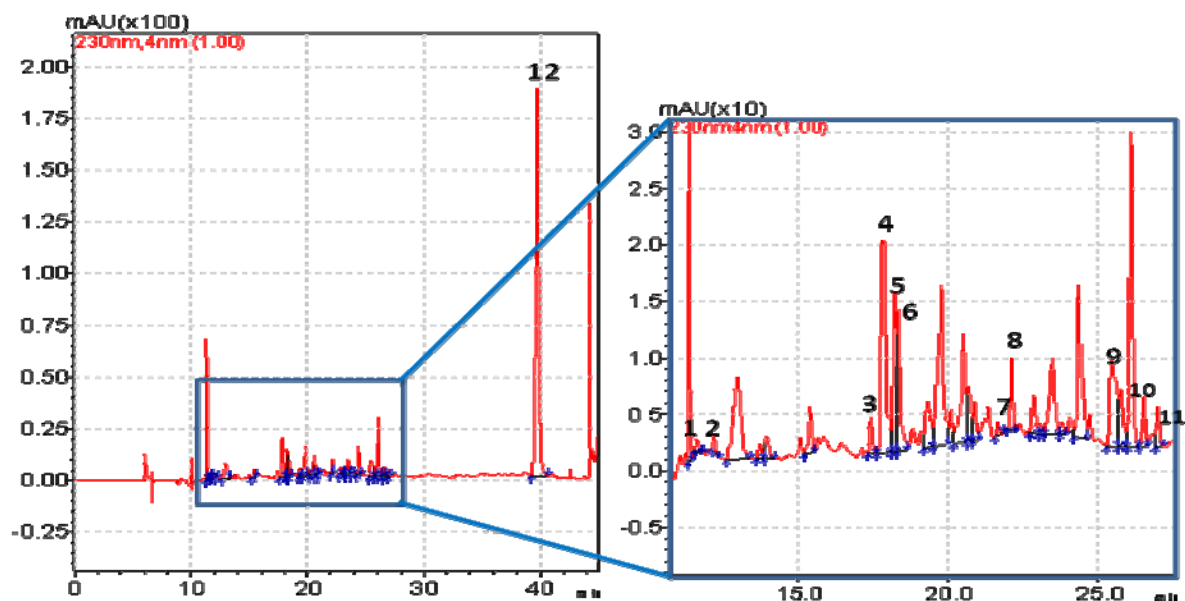


Fig. 3. UV chromatogram of the plasma sample in case 2: 1 = nordoxylamine, 2 = doxylamine (DA), 3 - 7 = amitriptyline-metabolites 8 = diphenhydramine (DPH), 9 = hydrocortisone, 10 = nortriptyline, 11 = amitriptyline and 12 = IS (N-ethyloxazepam).

For the quantification of amitriptyline and nortriptyline, calibration solutions in the range between 0 and 1.0 mg/L were prepared. Each calibration solution was measured three times ( $n = 3$ ). The function of the calibration curve for amitriptyline is:  $y = 17.17x$  ( $R^2 = 0.999$ ) and  $y = 9.40x$  ( $R^2 = 0.999$ , nortriptyline). Based on these functions, the amitriptyline and nortriptyline concentration in the patient was calculated: amitriptyline was found at 0.084 mg/L in the lower therapeutic range (0.05 - 0.2 mg/L); nortriptyline at 0.16 mg/L in the higher therapeutic concentration range (0.07 and 0.17 mg/L).

#### 4. Conclusion

A further method for the identification of weak basic, neutral and weak acidic compounds in human plasma was developed and integrated in the TOX.I.S.. Using the new method, the automated quantification of two model compounds (doxylamine and diphenhydramine) was successfully validated and applied successfully to more than 450 intoxications.

#### 5. References

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