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Electroanalytical overview: The detection of the molecule of murder atropine

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ABSTRACT

In this overview we explore the electroanalytical determination of the molecule of murder: atropine. Atropine, occurs naturally in various plants of the nightshade family, including the deadly nightshade (*Atropa belladonna*). On the one hand, atropine, a tropane alkaloid, has medical uses, named on the World Health Organisations list of essential medicines, used for example in resuscitations and as an antidote to certain poison gases and insecticides, but on the other hand, it is fatal in a high enough dose. Atropine derives its name from *atropos*, one of the three Fates, where in Greek mythology, one of the Fates determining the individuals moment of death. There is clearly a need to analytically determine atropine within clinical and other misdemeanours situations. In this overview, we review the current research directed to the electroanalytical sensing of atropine.

Introduction: atropine

Atropine, as shown in Scheme 1, is a naturally occurring belladonna alkaloid and is present in plants from the Solanaceae family. Atropine is a racemic mixture of equal parts of D- and L-hyoscyamine, whose activity is due almost entirely to the levo isomer of the drug. [1] Atropine has widespread central and peripheral actions and is named on the World Health Organisations list of essential medicines due to its important medical uses. [2] The principal effects of atropine are a reduction in bronchial, salivary, nasal, sweat and gastric secretions. [1] Atropine is also a muscarinic antagonist used to treat poisoning by muscarinic agents, including organophosphates or carbamate insecticides. [3] Atropine is destroyed by enzymatic hydrolysis, particularly in the liver and 13 to 50% is excreted unchanged in the urine. Atropine is given via inhalation for bronchodilation where serum levels of 2 to 6 ng/mL were detected after an elimination time of 4 h. [4] Atropine is reported to have a chemical blood therapeutic or normal range from 0.035–0.200 µg/mL with a lethal level reported to be above 0.2 µg/mL. [5]

While atropine is used to save lives, it is also used to take lives.¹ Atropine is a health and life-threatening alkaloid and its identification and quantitation are important in clinical and forensic toxicology.

[6–11] For example atropine has been found to be added into cocaine (10%) in the Netherlands [12] but one of the most intriguing cases of using atropine as a poison, is the case of Dr Paul Agutter in 1994 who attempted to murder his wife by spiking her favourite tippie of gin and tonic with atropine. [10] Since atropine has a bitter taste which can be detected as low as 100 ppm, Agutter added it to tonic water to mask its bitterness. Agutter purchased a dozen or so bottles of tonic water from his local supermarket in Edinburgh and spiked them with atropine, returning all but one bottle back onto the supermarket shelves. Agutter's idea was to cover his tracks by misleading the police into thinking that a criminal was trying to blackmail the supermarket and that Agutter's wife, who frequently shopped there, had unfortunately purchased a tampered bottle of tonic water. Agutter's wife ingested the atropine laced tonic (with gin) but found the drink too bitter and drank only part of it, had severe atropine poisoning and was treated at the local hospital. Fortunately, one of the ambulance team took possession of the bottles of drink and also poured the remaining of Mrs Agutter's gin and tonic into an empty jar which was later analysed. It was not only Agutter's wife who suffered, the poisoned tonic water from the supermarket affected eight people that week who were admitted to hospital suffering from symptoms of atropine poisoning. Agutter was found guilty of attempted

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¹ Atropine is related to the three Fates of Greek legend. Fates were personified as three very old women who spin the threads of human destiny, named Clotho (Spinner), Lachesis (Allotter), and Atropos (inflexible), the oldest of the three fates. Clotho spun the "thread" of human fate, Lachesis dispensed it, and Atropos cut the thread, determining the individual's moment of death. Atropos lends her name to the genus *Atropa*, of which the poisonous plant *Atropa belladonna* (deadly nightshade) is a member and to the alkaloid atropine.

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Table 1

An overview of electroanalytical approaches reported for the sensing of atropine.

Electrode	Electrode Modifier	Technique	Linear Range	Limit of Detection	Sample Medium	Reference
electrospun carbon nanofibers - CPE	Nafion- Ru(bpy) ₃ ²⁺	ECL	0.1 μ M – 0.1 mM	0.1 μ M	Spiked urine	[35]
GC	CNPs – Nafion- Ru(bpy) ₃ ²⁺	ECL	0.5 - 250 μ M	0.17 μ M	Injection sample	[50]
rGO–CPE	Co ₃ O ₄	DPV	0.1 – 3.2 μ M	0.03 μ M	Spiked urine and serum, injection sample.	[38]
Pt	PEDOT	LSV	0.1 - 0.8 μ M, 2.5 - 100 μ M	64 nM	Spiked urine	[51]
SPE	–	CV	5 μ M – 50 μ M	3.9 μ M	Cola-Cola	[45]
MWCNTs - CPE	SDBS	DPV	3.98 - 27.23 ng/mL	0.449 ng/mL	<i>D. stramonium</i> seeds, leaves and eye drops.	[41]
GC	SWCNTs/Chitosan	SWV	100 - 1000 nM, 5 - 150 μ M	16.5 nM	<i>D. stramonium</i> leaves, eye ointment, spiked human urine and serum.	[42]
PGE	ds-DNA/ PDDA–TiO ₂ NP–MWCNTs	DPV	0.6 - 30 μ M, 30 - 600 μ M	30 nM	Spiked serum and urine	[40]
GC	GSH–Co ₃ O ₄	DPV	0.01 – 0.46 μ M	0.001 μ M	Spiked urine	[39]
ITO	SMCs	ECL	0.2 – 82.9 μ M, 82.9 – 500.0 μ M	85 nM	Spiked serum	[52]
GC	Nafion/p-GNF	AdASV	6.0 μ M - 0.1 mM	1.9 μ M	Spiked urine	[53]
SPE	Ru(bpy) ₃ ²⁺ - Nafion film	ECL	0.75 - 100 μ M	0.75 μ M	Tomato and <i>Datura</i> Plants, Spiked Coca-Cola, tonic water	[36]
3D-graphene	Activated	SWV	5 - 60 μ M	1 μ M	Spiked vodka, white wine, whiskey, energy drink	[47]

Key: CPE: carbon paste electrode; GC: glassy carbon electrode; CNPs: carbon nanospheres; ECL: electrochemiluminescence; rGO: reduced graphene oxide; SPE: screen-printed electrodes; PEDOT: poly (3, 4-ethylene-dioxythiophene); DPV: differential pulse voltammetry; CV: cyclic voltammetry; AdASV: adsorptive anodic stripping voltammetry; LSV: linear sweep voltammetry; MWCNT-CPE: multi-walled carbon nanotube paste electrode; SDBS: sodium dodecyl benzene sulfonate; SWCNTs: single walled carbon nanotubes; MWCNTs: multi-walled carbon nanotubes; SWV: square wave voltammetry; Nafion/p-GNF: Nafion/polycarboxylate functionalized graphene nanoflakes; ITO: indium tin oxide; SMCs: vertically aligned silica mesochannels; PGE: pencil graphite electrode; PDDA: poly-diallyldimethylammonium chloride; TiO₂NPs: titanium dioxide nanoparticles; Pt: platinum electrode; GSH: glutathione.

murder in 1995 and after serving his punishment found employment lecturing at the University of Manchester on philosophy and ethics. A full account can be found in reference [10].

Clearly there is a need to identify and measure atropine, consequently, analytical methods for atropine determination include high performance liquid chromatography – UV, [13, 14] liquid chromatography–tandem mass spectrometry [12, 15] and liquid chromatography electrospray ionization tandem mass spectrometry to name just a few laboratory based approaches. [16] Although accurate, these lab-based methodologies require increased time for sampling, processing and analysis. On-the-spot testing for dangerous analytes such as this is key to the rapid treatment or prevention of ingestion. There have been reports of colorimetric and fluorometric approaches which can offer portability, however they can suffer in terms of selectivity and stability. [17–19] Electroanalytical approaches can offer portability with fast and reliable sensing whilst also offering high sensitivity and selectivity towards electroactive analytes. [20–22] In this review, we overview the recent approaches to the sensing of atropine and offer insights into where the field can be expanded.

Electroanalytical sensing of atropine

Table 1 provides a summary of the various electroanalytical approaches reported for the sensing of atropine which provide low limits of detection, micromolar down to even nanomolar and report to measure atropine in a range of sample matrixes; we overview some of these exciting reports.

Nearly all electrochemical reports start with polarography, which has of course been used to measure atropine as early as 1960, [23] as have potentiometric sensors. Vytřas has provided a summary of ion selective electrodes (ISE) [24] and for example, liquid membrane electrodes with *p*-nitrotoluene or *n*-octanol mediators and atropinium picrolonate or 5-nitrobarbiturate ion-pairs can provide a linear response towards atropine over the range of 10^{−5} to 10^{−2} M. These systems produced a limit of detection (LOD) of 5 μ M with response times between 3 - 90 s, with potential readings reported to be stable over the pH range 3–8. The authors applied their ISE to the successful determination of atropine in injection and eyedrop samples. Other ISEs have been

reported, [25–29] but in this review we shall not consider them further.

Hyphenated techniques have been reported such as using a glassy carbon macroelectrode coupled with high performance liquid chromatography to determine atropine in Belladonna powder. [30, 31] The authors reported that in the choice between electrochemical and spectrophotometric determination, the former offers a ten-fold increase in sensitivity and a wide linear response to atropine from 1 μ M to 5 mM owing to the low UV absorptivity of atropine. [30, 31] Gao and co-workers [32] used capillary electrophoresis (CE) with electrochemiluminescence (ECL) detection, using Ru(bpy)₃²⁺ for the simultaneous determination of atropine and scopolamine, the two major active ingredients in *Flos daturae*. Note that the concept of using CE-ECL with Ru(bpy)₃²⁺ has been well established but Gao and co-workers appear to be the first to extend this to atropine and scopolamine. The electrochemical detection was an end-column detection within a reservoir, employing a wall-jet configuration with a 500 μ m platinum disc, where a solution of 5 mM Ru(bpy)₃²⁺ and 50 mM phosphate buffer was utilised. Detection limits of 50 nM and 1 μ M for atropine and scopolamine respectively were reported to be possible, with the method successfully applied to determine the amounts of both alkaloids in *Flos daturae*, a traditional Chinese crude herb, which has been used as antitussive, antispasmodic and analgesic agents for thousands of years. [32] Yuan and co-workers have explored the simultaneous determination of atropine, anisodamine, and scopolamine by nonaqueous capillary electrophoresis coupled with electrochemiluminescence and electrochemical dual detection (ECD) using a platinum microelectrode (500 μ m diameter). [33] The authors found the electrochemical dual detection provided a slightly better LOD than that achievable using electrochemiluminescence, with the linear ranges of atropine, anisodamine, and scopolamine found to correspond to: 0.5–50, 5–2000, and 50–2000 μ M, respectively, with LODs of 0.5, 2 and 5 μ M using ECD, which also gave lower % Relative Standard Deviation than electrochemiluminescence. The methodology was shown to be viable to determine in scopolamine, atropine, and anisodamine in *F. daturae* plant extract. [33] Other similar work has been reported with the analysis extended to atropine injection samples and tablets. [34]

Xiu-Yun and co-workers developed an electrochemiluminescence (ECL) sensor based on tris(2,2'-bipyridyl) ruthenium(II) (Ru(bpy)₃²⁺)

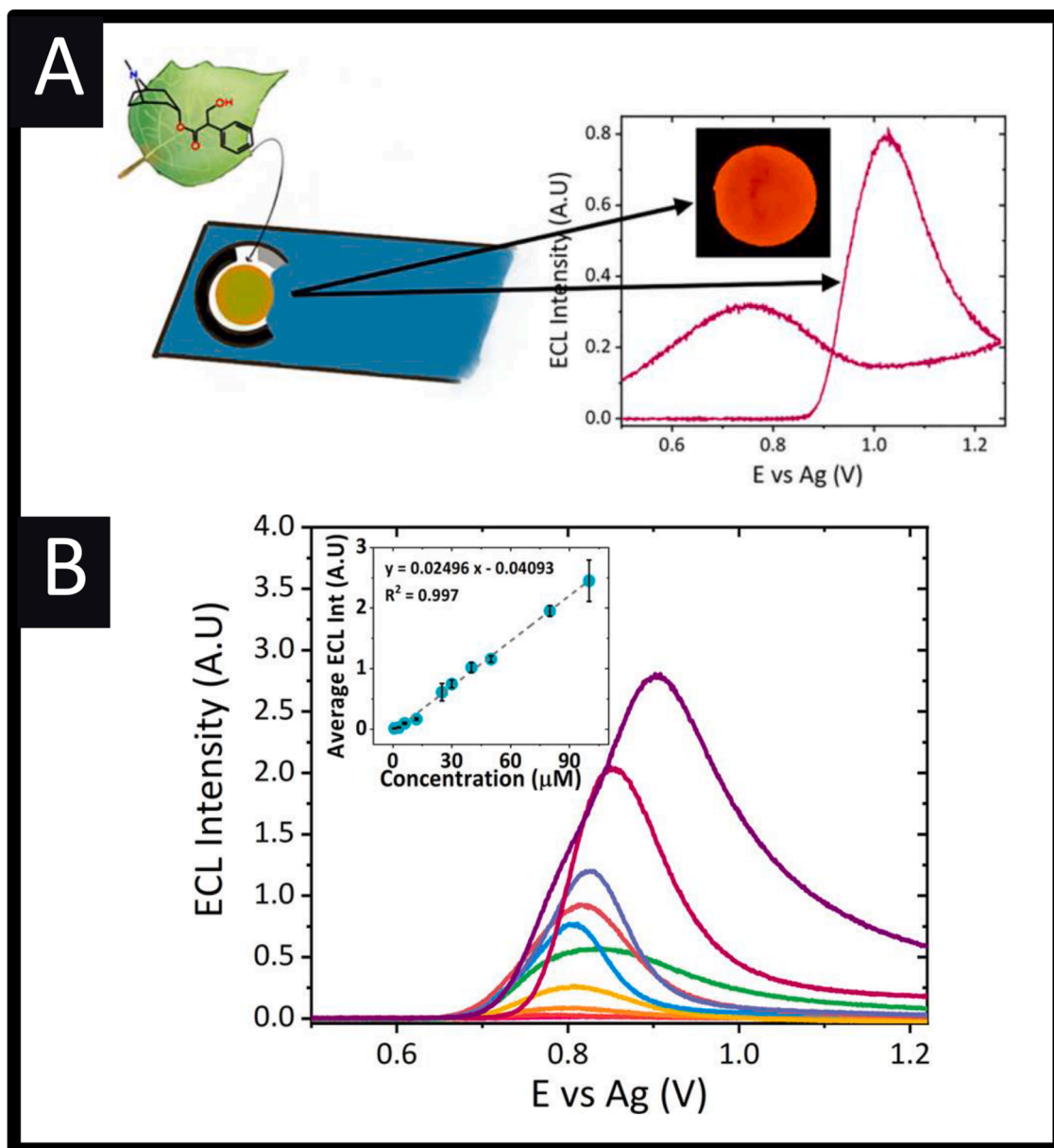


Fig. 1. A: Schematic overview of the use of screen-printed electrodes surface modified with a $[\text{Ru}(\text{bpy})_3]^{2+}$ - Nafion® film for the electrochemical luminescence sensing of atropine. The colour of the electrode shows schematically the change in the oxidation state of the ruthenium complex. B: Dependence of ECL signal on atropine sulfate concentration between 0.75 to 100 μM in pH 8 (0.1 M LiClO_4) at a scan rate of 100 mV s^{-1} across a potential range of +0.5 to +1.22 V vs. Ag at a PMT setting of +0.45 V. Inset shows the trend of maximum ECL signal against atropine sulfate, Figures reproduced from: [36].

for the sensing of atropine. [35] The sensor was fabricated by first constructing a carbon paste electrode, which is then modified via drop casting a mixture of electrospun carbon nanofibers and Nafion®. The resulting electrode is then immersed into a concentrated solution of $\text{Ru}(\text{bpy})_3^{2+}$, which is then rinsed with distilled water and then is ready to use. Since $\text{Ru}(\text{bpy})_3^{2+}$ is positively charged, it is easily incorporated into the electrospun carbon nanofibers and Nafion® film via electrostatic interactions, providing a simple electrode modification approach. The authors optimised the electroanalytical platform exploring the effect of pH, electrode surface composition and found via ECL that atropine could be detected over the range 0.1 μM – 0.1 mM, with a LOD of 0.1 μM and demonstrated the sensor to be successfully measure atropine in spiked urine with good recoveries (81–87%). [35]

Brown et al. [36] building upon earlier work extended the above to develop an ECL based sensor using a $\text{Ru}(\text{bpy})_3^{2+}$ - Nafion® film modified screen-printed electrodes. Fig. 1A shows a schematic of the electrodes

and typical ECL signal that is obtained when measuring atropine, note the colour change of the electrode surface as a result of the oxidation of the $\text{Ru}(\text{bpy})_3^{2+}$ complex; scheme 2 shows a potential mechanism of the ECL process for atropine sensing. Brown et al. found a linear dependence of the ECL signal upon atropine concentration from 0.75 to 100 μM , as shown in Fig. 1B, with a LOD of 0.75 μM . The authors demonstrated their sensor for the successful determination of atropine in spiked Coca-Cola and tonic water with good % recoveries (84–96%). Additionally, the authors extended their study to demonstrate their atropine sensor in the direct analysis of *Solanum lycopersicum* (tomato) and *Datura* plant extracts. The extracts were air-dried and placed into an aqueous solution adjusted to pH 8 via ultrasonication and analysed. Alternatively, a new method was reported where the samples were mechanically applied to the modified electrode, followed by modification of the surface with electrolyte to obtain the required conductance. Note that in these samples, scopolamine is also present with atropine and both have

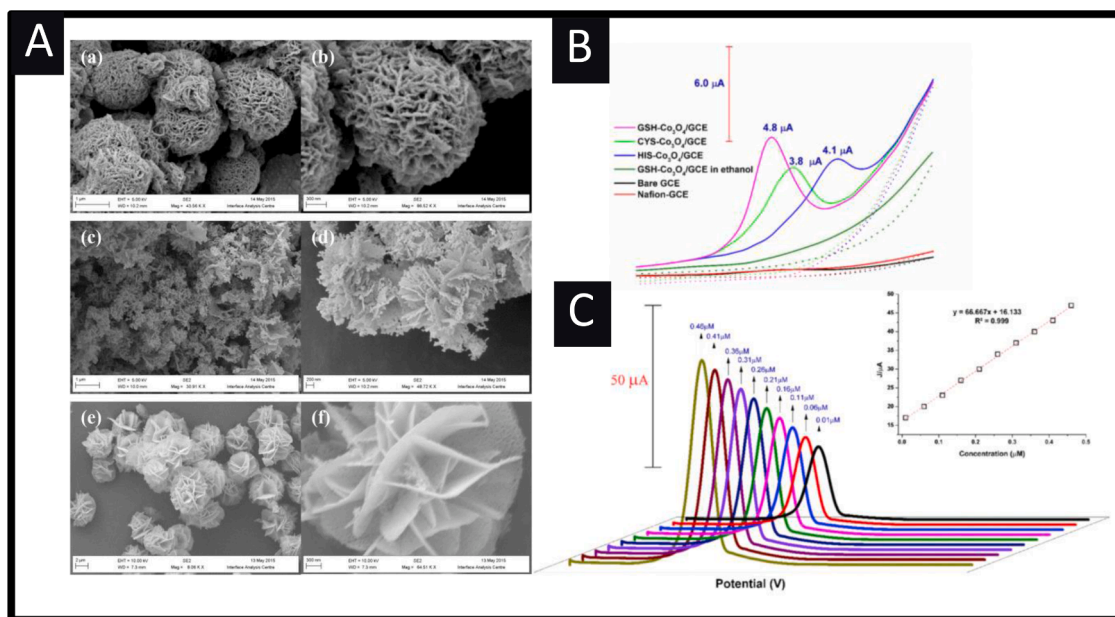


Fig. 2. A: SEM images for Co_3O_4 nanostructures grown using (a–b) cysteine (CYC) (c–d) histidine (HYS) and (e–f) glutathione (GSH) as effective growth template. B: The cyclic voltammetric responses of amino acid template Co_3O_4 nanostructures for $0.01 \mu\text{M}$ atropine within 0.1 BRB ($\text{pH } 10$) referenced against bare GCE and Nafion-coated GCE, and response of GSH- Co_3O_4 /GCE in the absence of atropine within 0.1 M ethanol solvent; C: Differential pulse voltammogram calibration plotted for GSH- Co_3O_4 /GCE against atropine concentration in range of 0.01 – $0.46 \mu\text{M}$ with inset graph portraying the corresponding linear fit analysis. [39].

identical electrochemical oxidation potentials, meaning a mixture of the two cannot be easily identified. To overcome this, *Solanum lycopersicum* (tomato) plant was used as a control, which doesn't contain atropine and scopolamine such that the ECL signal of the *Datura* plant extracts were subtracted from that of the *Solanum lycopersicum* (tomato) which provides an estimation of the total alkaloid components present in the *Datura* plant extracts. The authors were able to extend this study and through a simple change of pH, the ECL method is able to measure both atropine and scopolamine. [37]

Bagheri et al. [38] reported a Co_3O_4 nanoparticle – reduced graphene oxide bulk modified carbon paste electrode. In this approach the Co_3O_4 nanoparticle modified reduced graphene oxide (Co_3O_4 -rGO) was fabricated via a hydrothermal route, which is then made into an electrode by mixing carbon powder and the Co_3O_4 -rGO into a paste electrode. The authors demonstrated that the Co_3O_4 provides the origin of the improved electroanalytical response due to a large surface area and improved electron transfer rate compared to just a bare carbon electrode and rGO only modified carbon electrode. The sensor was able to measure atropine over the range 0.1 – $3.2 \mu\text{M}$ with an LOD of $0.03 \mu\text{M}$. The authors explored the interferents ascorbic acid, uric acid, glucose, lactose, sucrose and dopamine which were found to not interfere with the main electroanalytical signal. The authors validated their sensor in spiked urine, human serum and an atropine injection sample. They directly compared their sensor with independent HPLC, which confirmed their electroanalytical sensor to measure atropine at low micromolar in real samples, which hold promise to provide for a rapid, facile and sensitive detection and biomedical analysis of atropine. Based upon the success of Bagheri et al. [38], Co_3O_4 nanostructures have been fabricated via a low temperature hydrothermal method which utilised cysteine (CYS), glutathione (GSH) and histidine (HYS) as effective templates, resulting in porous flower-like shaped morphologies, as shown in Fig. 2A. [39] Fig. 2B shows a comparison of the various material morphologies modified upon a glassy carbon electrode and explored towards the measurement of atropine. This demonstrated that the GSH- Co_3O_4 gives rise to the largest signal at the lowest overpotential, which the authors attribute to the enhanced electron transfer kinetics and higher electrochemical reactivity associated with the unique structural features of flower-like Co_3O_4 . Fig. 2C shows the electroanalytical signals and

resulting calibration plot using the GSH- Co_3O_4 modified glassy carbon electrode which gave rise to a linear range of 0.01 – $0.46 \mu\text{M}$ with a LOD of $0.001 \mu\text{M}$. The authors explored the potential interferents glucose, fructose, cysteine, uric acid, dopamine and ascorbic acid which were found to have no effect on the sensing of atropine and went on to demonstrate the successful determination of atropine in spiked urine. [39]

Ensafi and co-workers [40] developed a biosensor based on a pencil graphite electrode (PGE) which is modified with multiwall carbon nanotubes (MWCNTs), titanium dioxide nanoparticles (TiO_2NPs), poly-diallyldimethylammonium chloride (PDDA) and decorated with ds-DNA (ds-DNA-PDDA- TiO_2NPs -MWCNTs/PGE). The PGE is literally as the name suggests, a pencil, and serves as cheap electrode which is easily renewed and modified. The ds-DNA-PDDA- TiO_2NPs -MWCNTs/PGE electrode was found to measure atropine over two linear ranges, 0.6 – $30 \mu\text{M}$ and 30 – $600 \mu\text{M}$ with a very low LOD of 30 nM reported. The authors validated their atropine sensor in spiked blood serum and urine and with independent HPLC confirming the sensor has potential to be up-taken to routinely measure real samples. [40]

Dar and co-workers [41] reported the use of a multi-walled carbon nanotube bulk modified paste electrode. The electrode is then modified with the anionic surfactant, sodium dodecyl benzene sulfonate, with the latter providing an enhancement in the electroanalytical performance. The reason for the improvement was attributed to the surfactant increasing the adsorption of atropine onto the multi-walled carbon nanotubes; consequently, the authors utilised differential pulse voltammetry with an accumulation time of 2 mins . The authors proposed a mechanism based on a 2 electron and 1 proton process and reported the electroanalytical determination of atropine from 3.98 ng/mL to 27.23 ng/mL with a LOD of 0.449 ng/mL . An interference study was explored with the following interferents: ascorbic acid, ofloxacin, norfloxacin, gatifloxacin, glucose, lactose with their concentrations 300 – 400 fold greater than atropine and yet still didn't interfere with the electroanalytical signal of atropine, with signal changes all below 5% . The authors demonstrated their sensor to determine atropine in *D. Stramonium* seeds and leaves and eye drops with high recoveries.

Extending the theme of carbon nanotubes, Mane et al. [42] utilised

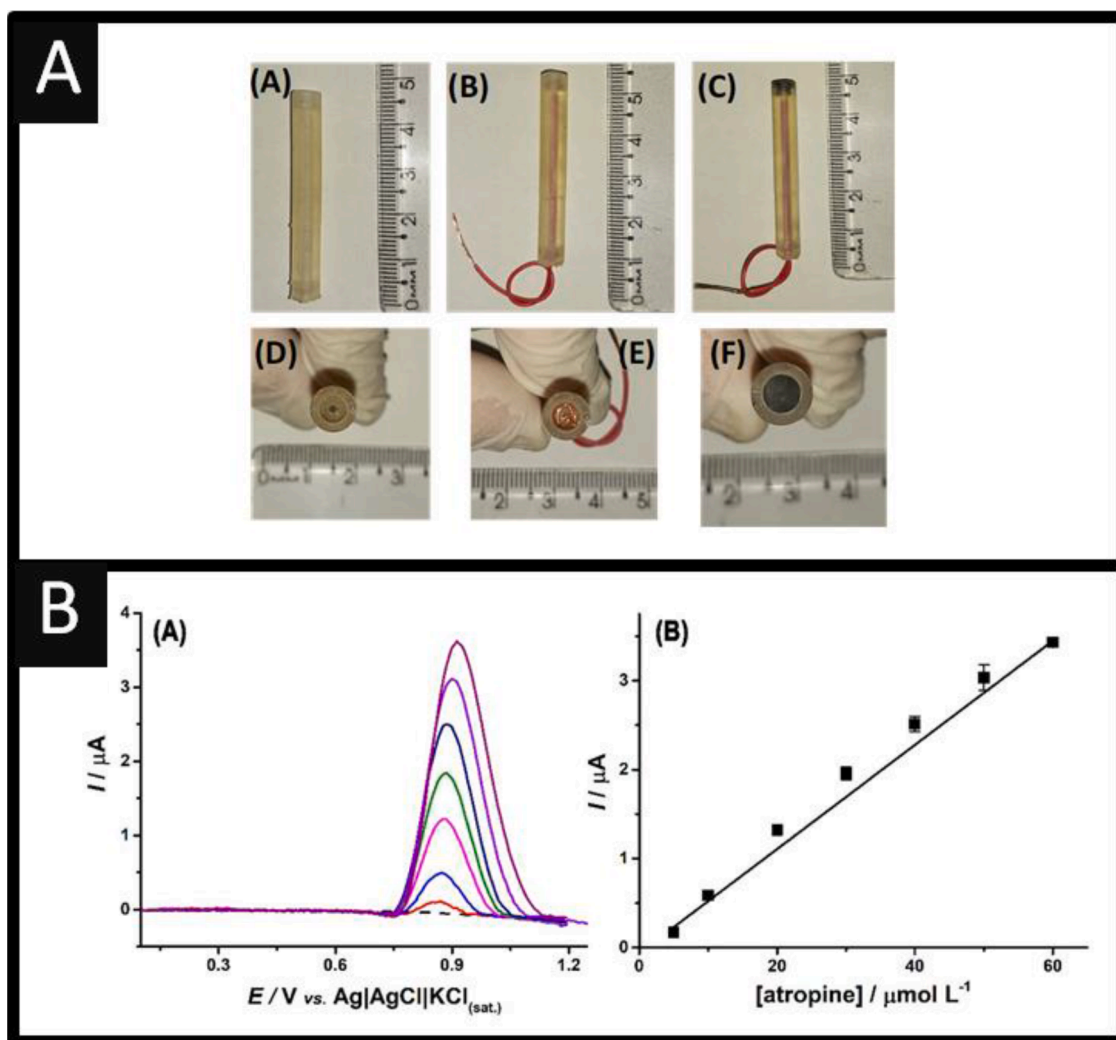
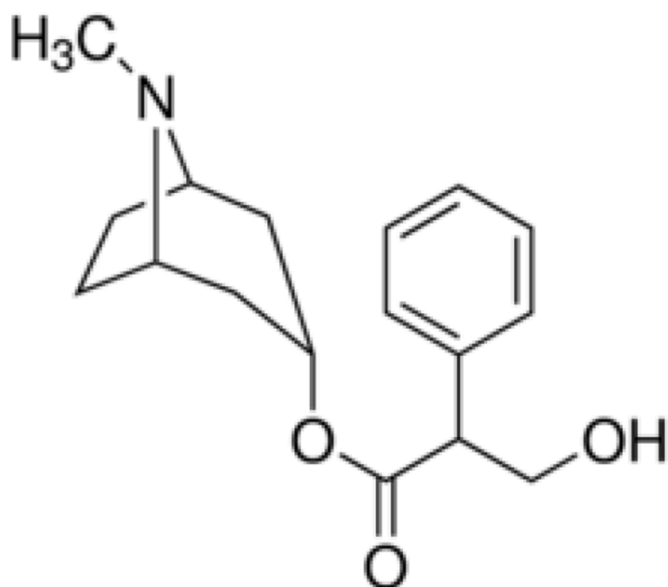


Fig. 3. A: (A, D) Printed acrylic cylindrical substrate. (B, E) Electrical contact is made by copper wire that was inserted into the holes of printed acrylic cylindrical substrate. (C, F) 3D printed electrodes obtained by filling the hole of the acrylic cylindrical substrate with conductive thermoplastic employing the 3D pen. B: Baseline-corrected SWV responses ($n = 3$) for increasing concentrations of atropine ($5\text{--}60\ \mu\text{mol L}^{-1}$) in pH 11 and (B) respective calibration curve. Reproduced with permission from Ref [47] Copyright Elsevier 2001.

single walled carbon nanotubes (SWCNTs) immobilised upon a chitosan film, coated upon a glassy carbon electrode. The authors reported that the peak current at the modified electrode was ~ 7.5 times higher compared to that at the bare glassy carbon electrode, which they attributed to the synergistic effect of SWCNTs and chitosan, accelerating the electron transfer in the bulk solution to the electrode surface resulting in a substantial change in the current response of atropine. [42] The sensor was demonstrated to measure atropine over two linear ranges $100\text{--}1000\text{ nM}$ and $5\text{--}150\ \mu\text{M}$ with a low LOD of 16.5 nM . The sensor was shown to successfully determine atropine in *D. stramonium* leaves, eye ointment, spiked human urine and serum. Trends throughout the literature on the electroanalytical detection of atropine point toward the use of nanomaterials to improve the platform performance, with many using carbon nanotubes. We suggest there is further scope for utilising different nanomaterials such as nanoparticles and metallic oxides [43] including the use of metal-organic frameworks (MOFs) [44].

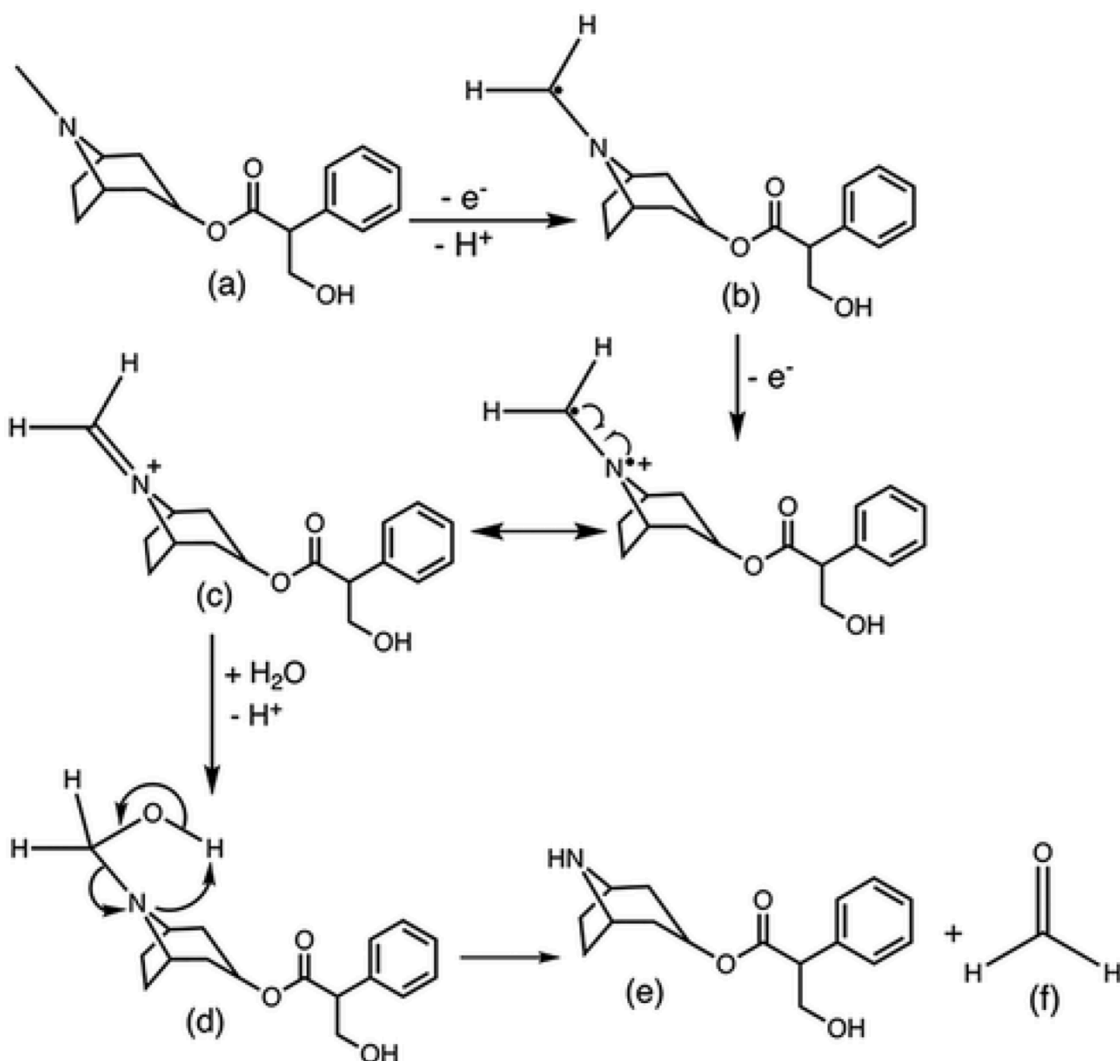
Ramdani et al. [45] explored the detection of atropine using screen-printed graphite macroelectrodes, which was shown to exhibit a linear range from $5\ \mu\text{M}$ to $50\ \mu\text{M}$ with a LOD of $3.9\ \mu\text{M}$. Screen-printed electrochemical platforms are mass-producible, resulting in low-cost sensors and yet are highly reproducible and versatile as they can be used “as is” or as the basis to electrically wire a range of nano and micromaterials used to detect the target analyte. [20] The authors

explored the interferents caffeine and ascorbic acid, commonly found in drinks samples, which were found to not interfere with the sensing of atropine. The authors demonstrated that atropine could be detected in diet Coca-Cola (following sui 1 dilution with buffer solution). An alternative to screen-printing is the ability to use additive manufacturing to produce electrochemical sensors. [46] To this end, João and co-workers [47] utilised a 3D printable pen to fabricate additive manufactured atropine sensors. As shown in Fig. 3, first an acrylic cylinder is printed with copper wire inserts to allow electrical contact. Next, the hole is filled via the printing with a 3D pen, using commercially available graphene doped PLA. Excess plastic is removed with an electric mini drill equipped with sand-paper which was then polished using abrasive paper. In order to activate the electrode, exposing the active sites (graphene), electrochemical activation (electrochemical potential cycling) was applied prior to the sensor being ready for use. As shown in Figure 3, square-wave voltammetry was used to analytically characterise the additive manufactured sensor, which gave rise to a linear range from 5 to $60\ \mu\text{M}$ with a LOD found to be $1\ \mu\text{M}$. Investigation of the mechanism indicated that it involved the transfer of two electrons and one proton, in agreement with prior literature reports. [45] In terms of over-viewing the endeavours to the electroanalytical sensing of atropine, the mechanism is widely variable suggesting a strong dependence upon electrode surface morphology and composition; further work should be directed to



Scheme 1. Chemical structure of atropine.

the unambiguous determination of the electrochemical mechanism of atropine via electrochemistry coupled with mass-spectrometry. João and co-workers [47] went on to demonstrate its viability in spiked vodka, white wine, whiskey and an energy drink, reporting good recovery values. Interestingly, in these real samples, pre-peaks were observed, which were generally resolved from the electroanalytical peak of interest (atropine), all except in the case of the vodka and whiskey samples where an additional peak was observed on the side of the main peak, arising from the real sample, but good recovery values were still obtained. The origin of these peaks were not identified. However, selectivity was further explored with the effect of diazepam, benzodiazepine, caffeine and glucose, which posed no interference. The low-cost of a 3D pen (less than 25\$) and each sensor estimated to be ~ \$0.10, with the usefully electroanalytical performance towards atropine sensing provides an attractive option for on-site determination. One possible method of overcoming future problems with interferents and matrix effects is through the use of molecularly imprinted polymers (MIPs). MIPs offer cavities for binding specific in size, shape and functionality for the target analyte, thereby offering the ability to trap the target at the surface while interferents could be washed off or removed. [48] We note that for colourimetric and fluorometric sensors mentioned previously, there is a trend in the incorporation of MIPs to help improve the selectivity of their systems. [17–19] There is scope for future research in this area for the electrochemical detection of atropine due to the facile synthesis of MIPs onto electrode surfaces. [49]



Scheme 2. Schematic detailing the electrolytic N-Dealkylation mechanism for atropine sensing during the ECL Process. Reproduced from: [36].

Conclusions and outlook

We have overviewed the recent work into developing electroanalytical based atropine sensors and while endeavours not overly massive, there are some promising reports that are able to sense atropine in biological samples. They have been validated against traditional laboratory based analytical techniques and hold promise for in-the-field electroanalytical based sensors, alleviating the need for laboratory-based measurements. Future work is needed to unambiguously determine the electrochemical mechanism of atropine determination, via electrochemistry-mass spectrometry. More work into developing selective sensors in plant-based media, where atropine and scopolamine are both encountered, and where their electroanalytical signals overlap, is also suggested to advance the field further.

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