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Evaluation of diazotized 4-amino-3,5-dinitrobenzoic acid (ADBA) as a new derivatizing reagent

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Summary

A preliminary evaluation of the reactivity of diazotized 4-amino-3,5-dinitrobenzoic acid (ADBA) towards selected aromatic compounds has been described. Successful diazo coupling of pharmaceuticals possessing aromatic rings of varying reactivities was achieved with the arenediazonium ion of ADBA at room (30°C) or elevated temperature (80°C). The adducts formed in spot-test reactions were coloured for some compounds (e.g, cloxacillin and chloroxylenol), others showed colour at elevated temperature of reaction (e.g., salicylic acid and aspirin), while others showed no detectable change in colour of reaction mixture, even at elevated temperature (e.g., imidazole and tinidazole). The coloured product formed at room temperature decomposed and the colour discharged at elevated temperature in some cases (e.g., β-naphthol). However, thin layer chromatographic analysis suggested that a more lipophilic derivative, relative to the original compound was formed for some of the compounds studied which did not show any detectable colour change in the spot test reactions. The diazotized ADBA is thus shown to be a reactive coupling reagent with which a suitable derivatization methodology could be developed for a wide range of pharmaceuticals in ultraviolet/visible spectrophotometry and high performance liquid chromatographic (HPLC) analysis.

Keywords: *Diazotized 4-amino-3,5-dinitrobenzoic acid (ADBA), reactivity, spot-test reaction, chromatographic analysis, aromatic compounds.*

Résumé

Une evaluation preliminaire de la reactivite de l'acide 4-amino-3, 5-dinitrobenzoique diatolise (ADBA) enver les compses aromatiques selectionnes ont ete deens. La resussite du couple diato pharmaceutiques possidant les anneaux aromatises de reactivites variables a ete accomplie a l'aide de l'aide de l'arenediazonium de ADBA a temperature ambriante (30°C) on temperature elevee (80°C). Les boules formies par les reactions au point test etaient colorees pour certain composes (exemple cloxacilline et chloroxylenol), les autres montraient une coloration a des temperatures de reaction tres elevees (exemple l'acide solicyclique et l'aspirine) alors d'antres ni montraivent ancum changement detectable en conleur aux reaction mixtes, meme a des temperature elevees (exemple imidazole et trinidazole). Le produit colore forme a temperature ambiante se decompresait et la couleur dechargee a temperature elevee dans certain cas (exemple β-naphthol) cependant, l'analyse chromatigraphique des plagues mines suggerait qu'une grande den'ree lipophilique relative an compose original s'etait formee pour certain des composes etudies, lesguels non montre un changement detectable de conleur sur les point de reation testes. L'ADBA diazole est done un reactif couple avec lequel une methodologie de derivatisation converiable pent etre developee pour un bon nombre de produits pharmaceutique en ultraviolet/spectrophotometre visible et l'analyse chromato graphique liquide a performance elevee (HPLC).

Introduction

Nitrophenyl and dinitrophenyl derivatives are particularly useful as derivatizing reagents for sensitivity enhancement of analytical methods in ultraviolet/visible spectrophotometry and high performance liquid chromatographic (HPLC) analysis with UV detection. This is due to the good absorptivity they afford and the stable derivatives they form [1]. For example, 2,4-dinitrophenylhydrazine (2,4-DNP) has been applied for carbonyl derivatization in the spectrophotometric assay of methyltestosterone tablets [2] and HPLC analysis of glyoxylic acid obtained from allantoin in biological fluids [3].

Similarly, 2-nitrophenylhydrazine has been applied for the determination of carboxylic acids as their respective hydrazides in colorimetric analysis of carboxylic acids [4] and HPLC analysis of saponified fatty acids of phospholipids [5].

In our previous work [6] the development of a new dinitrophenyl derivative, 4-amino-3,5-dinitrobenzoic acid (ADBA) as a novel derivatizing reagent for aromatic ring derivatization *via* diazo coupling reaction was reported. Diazotized ADBA was demonstrated to be highly reactive and capable of forming azo compounds with aromatic ring of varying reactivities. Successful coupling was demonstrated with βxnaphthol, phenanthrene and halofantrine representing aromatic compounds with activated, neutral and deactivated nuclei respectively [6].

In this paper we report a preliminary evaluation of the reactivity of diazotized ADBA with a wider range of pharmaceuticals possessing aromatic nuclei of varying reactivities. The scope of work includes spot-test reaction and subsequent thin layer chromatographic analysis of coupling reaction mixture.

Materials and methods.

Reagents

Glacial acetic acid, methanol, ethyl acetate, petroleum spirit (60-80°c), liquid paraffin, magnesium sulphate, sodium hydrogen carbonate, sodium hydroxide pellets (British Drug Houses (BDH)). Pure samples ran on the thin layer chromatography include: metronidazole, imidazole, tinidazole, miconazole, chloroquine, anodiaquine, quinoline, cloxacillin, ampicillin, lignocaine, chloroxylenol (Sigma, U.K),βx-naphthol, aspirin, salicylic acid, (BDH, UK), silica gel G F_{254} (Merck, Germany) and 4-amino-3,5-dinitrobenzoic acid (ADBA) (synthesized in our laboratory).

Equipment

Analytical balance (Mettler AE 160, Mettler PC 400),

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ultraviolet lamp (254 and 365nm), thin layer spreading apparatus, thermostated water bath (Buchi; Switzerland).

Preparation of diazotized ADBA reagent solution
The solution of ADBA in concentrated sulphuric acid was diazotized with sodium nitrite using our previously reported procedure [6].

Preparation of stock solution of compounds
Each compound (10mg) was dissolved in 1ml of glacial acetic acid.

Coupling reaction
Each compound stock solution (0.2ml) was added to a test tube containing 0.5ml of the reagent solution. The test tube was shaken vigorously and the colour produced was observed within 5 minutes and recorded.

Effect of temperature on coupling reaction
Each reaction mixture was heated in a thermostated water-bath at 80°C for 30 minutes. Any change in colour on heating or change in the intensity of colour in each tube was observed and recorded. A control reaction tube was set up, containing the reagent solution alone.

Processing of the reaction mixture
The reaction mixture was transferred into an ice-bath from the water-bath after 30 minutes. Ice-cold water (5ml) was added to the test-tube, shaken vigorously and extracted with 3 x 2ml of ethyl acetate with 50 inversions of the tube at each extraction. Dilute solution of sodium hydrogen carbonate was added drop wise to the combined ethyl acetate extract.The ethyl acetate phase was separated and dried over anhydrous magnesium sulphate.The extract was filtered and concentrated to a small volume on a water-bath. The same procedure was repeated for each drug as well as the control. The concentrated extract was reserved for thin layer chromatographic analysis.

Thin layer chromatographic (TLC) procedure
The thin layer chromatographic analysis involved the preparation of plates, sample solutions for spotting, development of plates and visualisation procedures.

Preparation of TLC plates
TLC plates were prepared for both normal and reversed phase chromatography.

Preparation of normal phase TLC plates
A slurry of silica gel GF_{254} (Merck) was made in water in a ratio of 1:2 (silica gel:water) and spread on 5x10cm glass plates with a layer thickness of 0.5mm. The plates were air-dried for 30minutes and activated at 125°C for 4 hours. Activated plates were stored in dessicator before use.

Preparation of reversed phase TLC plates.
Normal phase plates prepared as described above were treated by ascending development in a tank containing 10ml of 5% liquid paraffin in petroleum ether (60-80°C, BDH). Developed plates were allowed to air-dry leaving plates impregnated with a thin film of liquid paraffin. Impregnated plates were stored in a dessicator before use.

Mobile phase
Mobile phase was optimized for both normal and reversed phase TLC analysis. For normal phase, the optimum mobile phase was ethylacetate/methanol (90:10), while for reversed phase, the optimum mobile phase is methanol/water (80:20).

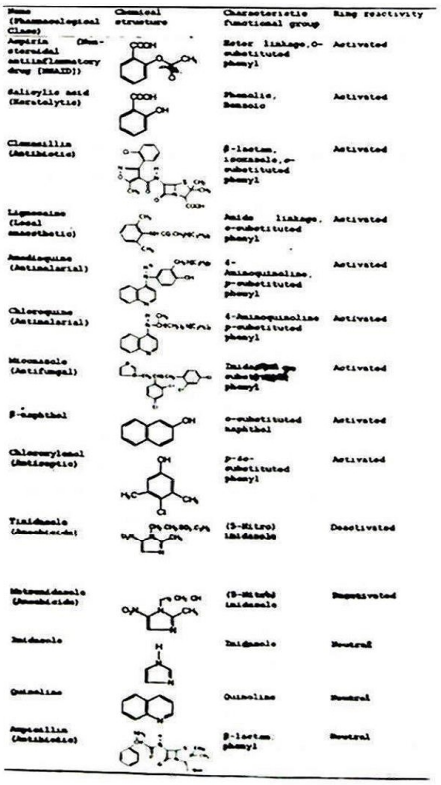
Sample spotting and development of chromatoplate
Normal and reversed phase TLC analyses were run for each of the compounds. For each compound, three different solutions were spotted. Firstly, ethyl acetate extract of reagent reaction mixture, secondly, solution of compound in methanol and lastly, ethyl acetate extract of control solution (diazotized ADBA). The spots were applied with capillary tube and allowed to air dry at room temperature.The spotted plates were developed in the saturated tank containing the appropriate solvent system for each mode of TLC analysis. The developed plates were air dried and visualised.

Visualization
The TLC plates (normal and reversed) for each compound were observed in daylight for coloured compounds and also under ultra-violet lamp at 254nm. Every spot observed under the UV light was identified and the respective R_f values recorded.

Results

Spot-test reactions
Diazo coupling reaction was carried out as described between diazotized ADBA and a number of aromatic compounds. The structure of compounds studied and their characteristic functional groups are shown in Table 1. The compounds are

Table 1: Chemical structures of study compounds.



classified as activated, neutral and deactivated, according to the reactiviy of the aromatic rings present towards electrophilic substitution reaction, which in turn is dependent on the nature of substituents on the ring. The reaction was carried out at room temperature (30°C) and at 80°C for 30 minutes. The result is shown in Table 2.

Table 2: Effect of temperature on coupling reaction

Compound	Colour of reaction mixture* Room temp (30°C)	80°C, 30 minutes
1. Imidazole	Light yellow	No change
2. Tinidazole	Light yellow	No change
3. Miconazole	Light yellow	No change
4. Metronidazole	Light yellow	No change
5. Aspirin	Light yellow	Dark brown
6. Salicylic acid	Light yellow	Dark brown
7. Ampicillin	Light yellow	No change.
8. Cloxacillin	Deep yellow (instantly)	No change
9. Chloroquine	Light yellow	No change
10. Amodiaquine	Light yellow	No change
11. Chloroxylenol	Orange (instantly)	More intense
12. Quinoline	Golden yellow (instant)	More intense
13. Lignocaine	Light yellow	No change
14. β-Naphthol	Blood red (instantly)	Dark brown

**The blank reagent solution without any coupling component is light yellow.*

Chromatographic analysis of reaction mixture.

Chromatographic analysis of the reaction mixture after coupling at 80°C for 30 minutes was carried out on both normal phase and reversed phase modes. Table 3 shows the properties of the ADBA-azo adduct formed with compounds under investigation.

Table 3: Properties of the ADBA-azo adduct formed with study compounds.

Presumed azo adduct with	Stationary Phase	Colour on TLC plates Daylight	UV-254	Rf
1. Imidazole	NP	Colourless	Purple	0.72
2. Tinidazole	NP	Colourless	Purple	0.68
3. Miconazole	NP	Colourless	Purple	0.68
4. Metronidazole	NP	Colourless	Purple	0.73
5. Cloxacillin	NP	Orange	Purple	0.66
6. Ampicillin	NP	Colourless	Purple	0.69
7. Chloroquine	NP	Colourless	Purple	0.69
8. Amodiaquine	NP	Colourless	Purple	0.80
9. Quinoline	NP	Orange	Dark brown	0.70
10. Chloroxylenol	NP	Colourness	Purple	0.82
11. Aspirin	NP	Colourless	Purple	0.75
12. Salicylic acid	NP	Colourless	Purple	0.75
13. Lignocaine	NP	Colourless	Purple	0.63
14. β-Naphthol	NP	Orange	Dark brown	0.82

**NP = Normal phase.*

Discussion

Coupling reaction

From the results shown in Table 2, the compounds containing the imidazole ring as well as the compounds with acid-labile linkages, e.g., aspirin, and lignocaine showed no colour at room temperature. Three compounds viz; β-naphthol, cloxacillin and chloroxylenol all gave instant colour. The effect of ring substituents on reactivity towards diazo coupling is exemplified by the β-lactam antibiotics studied. The phenyl ring in ampicillin is unsubstituted and showed no colour at room temperature, while cloxacillin with mildly activating chlorine substituent gave instant colour at room temperature. Due to the isoxazole heterocycle substituent in cloxacillin the activation of the phenyl ring was enhanced. All the compounds containing deactivated rings and neutral rings gave no colour at room temperature. However, quinoline which was classified as a neutral ring because it was devoid of ring substituents, gave instant colour because it was more reactive towards diazo coupling reaction, owing to its polycyclic nature.

Effect of temperature on coupling reaction

The coupling reaction mixture was subjected to heating at 80°C for 30 minutes, in order to investigate the effect of temperature on coupling reaction. The results are as shown in Table 2. Application of heat affected the appearance of the reaction mixture for some compounds. Salicylic acid and aspirin gave dark brown colour with salicylic acid giving a relatively darker shade. Chloroxylenol and quinoline reaction mixture became more intense, while the rest were unaffected by heat. Typically, diazo coupling reaction is carried out at 0°C because of the thermolability of arenediazonium ions [7]. However, coupling at 80°C for 30 minutes is possible with diazotized ADBA because it exhibits an unusual thermostability in the acidic medium employed for its preparation [6].

The colour of the adduct and the tlc profile were the same for aspirin and salicylic acid, suggesting that aspirin first underwent acid hydrolysis on heating.The hydrolytic product formed, salicylic acid, being more reactive toward coupling reaction (activated ring) gave rise to progressive colour formation with time. The greater colour intensity obtained with chloroxylenol and quinoline on heating was due to increased rate of reaction produced by temperature rise.

However, the blood red colour formed with β-naphthol at room temperature was discharged at elevated temperature to form a dark brown solution. This is indicative of thermal degradation of the azo derivative previously formed.

Chromatographic analysis of coupling reaction mixture

Chromatographic analysis of reaction mixture was carried out to complement the observations made in spot-test reactions. This afforded detection of the presence of adducts with faint colour intensity which was not easily distinguishable from the light yellow colour of the blank reagent. The intensity of an azo adduct is a function of the extent of conjugation. This will vary from one compound to the other.

Both normal and reversed phase tlc were employed for the analysis in order to ascertain the relative merit of the two stationary phases in resolving the components of a typical coupling reaction mixture. This was designed as a prelude to the application of the reagent as a pre-column derivatizing reagent in high performance liquid chromatographic (HPLC) analysis. The chromatoplate was visualized in daylight and under uv light (254 nm) after development. Table 3 shows the properties of the presumed azo adduct formed between ADBA and the compounds. Generally, the resolution was better on the normal phase than the reversed phase tlc. The presumed azo adduct has the highest R_f in normal phase tlc, while in the reversed phase it is often found at the origin. Although some compounds did not show any coloured products after coupling, all the compounds studied produced additional compounds different from the residual reagent and unreacted reactants. The R_f value obtained for such additional compounds often suggests that the compound is lipophilic, as expected of an azo adduct relative to the two reactants. However, it is uncertain if azo adducts are actually formed, since possible formation of other adducts cannot be ruled out.

Multiple products were found on tlc for the chloroxylenol reaction mixture. This is indicative of thermal

decomposition during application of heat. The 4-aminoquinolines studied (chloroquine and amodiaquine) gave no colour in the spot test reaction even with application of heat, whereas quinoline gave instant orange colour which deepened with time. The tlc analysis showed the presence of other products with R_f values suggestive of an adduct of the reagent and the aminoquinolines. Yet, the absence of colour in the spot test reaction is sufficient evidence that formation of C-azo adduct could not have occurred with the 4-aminoquinolines. This observation is consistent with the findings of Renshaw *et.al* [8] which reported that 4-aminoquinolines failed to couple with benzenediazonium chloride under any coupling condition tried. On the other hand, other substituted aminoquinolines coupled at different positions. It would appear from the report that position 4 (*para*-position) has to be vacant for the quinoline ring to undergo C-azo coupling reaction. Similarly, in this present work, all the compounds that formed coloured adduct have vacant *para*-positions.

Overall, the results obtained suggest diazotized ADBA is a reactive reagent applicable to a wide range of pharmaceuticals and aromatic compounds of varying reactivities. The coupling reaction could be adapted by validation and optimization studies to develop appropriate derivatization methodology for the spectrophotometric analysis and high performance liquid chromatographic (HPLC) analysis of the prospective compounds.

Conclusion

The arenediazonium ion, diazotized 4-amino-3,5-dinitrobenzoic acid (ADBA), shows high reactivity towards diazo coupling reaction with aromatic rings of varying reactivities. Compounds with activated rings form coloured adducts instantly, presumably with formation of C-azo adducts. A compound with ester linkage behaved like precursor of its more reactive hydrolytic product which more readily undergoes coupling reaction. Chromatographic analysis of coupling reaction mixture suggests that formation of adducts possibly occur for many compounds that show no visible colour reaction. Further work is on going in our laboratories to extend this coupling reaction to optimization and validation studies for development of new method of assay for the promising compounds *via* this derivatization technique in spectrophotometric and HPLC analysis.

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