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# ELECTROCHEMICAL DETECTION OF SYMPATOMIMETIC DRUGS, FOLLOWING PRE-COLUMN *o*-PHTHALALDEHYDE DERIVATIZATION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

In order to assay sympatomimetic drugs (heptaminol and related compounds), a derivatization procedure with the use of *o*-phthalaldehyde (OPA) and various thiols was developed. Some structural assignments of the OPA–amino drug adducts are proposed and the electroactive properties of these substituted isoindolic products were investigated by voltamperometry. High-performance liquid chromatographic separations were carried out on a reversed-phase column (LiChrosorb RP-18) with phosphate buffer (pH 7.2)–methanol mixtures as mobile phase. For detection , either UV spectrophotometry ($\lambda$ = 340 nm) or amperometry (+0.9 V) was used. By the latter levels in the picomole range were detectable.

## INTRODUCTION

Assays of sympatomimetic drugs in biological fluids usually rely upon gas chromatographic (GC) enzyme multiplied immunoassay technique (EMIT) and radioimmunoassay (RIA) methods. The sensitivities of these methods have recently been compared[1]. Direct high-performance liquid chromatographic (HPLC) methods do not allow measurement of these compounds at low levels owing to their very weak UV absorption properties. Several derivatization procedures for spectrophotometric or fluorimetric detection in HPLC systems have been investigated[2].

In order to enhance the sensitivity of measurement, electrochemical detection may be envisaged after the derivatization of amino drugs to form electroactive species. Usual electrophores are nitro groups[3], which are easily reduced at a dropping mercury electrode. However, a reductive method must be applied with great care in a HPLC system with electrochemical detection. A derivatization procedure with oxidizable electrophores for primary and secondary amines has already been described[4], but the reaction involves no commercially available reagents.

Since the first report by Roth[5] and the further studies by Simons and Johnson[6,7], *o*-phthalaldehyde (OPA) has proved its efficiency in the analysis of amino acids[8,9], biogenic amines[10] and drugs[2,11] and drugs in either the pre- or post-column

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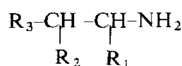
TABLE I

FORMULAE OF THE STUDIED SYMPATOMIMETIC DRUGS




| *Drug* | $R_1$ | $R_2$ | $R_3$ | *Form* | *N.W.* | *Source* |
|---|---|---|---|---|---|---|
| Heptaminol | $CH_3$ | H | $(CH_3)_2=C(OH)-(CH_2)_2-$ | Hydrochloride | 181.7 | * |
| Amphetamine | $CH_3$ | H | $C_6H_5$ | Sulphate | 368.5 | ** |
| Norephedrine | $CH_3$ | OH | $C_6H_5$ | Hydrochloride | 187.7 | *** |
| Phenethylamine | H | H | $C_6H_5$ | Base | 121.2 | *** |
| 2-Heptylamine | $CH_3$ | H | $CH_3-(CH_2)_3$ | Base | 115.2 | *** |

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derivatization mode with fluorimetric detection. We have recently developed a pre-column derivatization procedure with the use of OPA for the assay of heptaminol in pharmaceutical preparations by HPLC with UV detection, which affords sufficient sensitivity for this purpose[12]. However, we have found, by analogy with indolic compounds[13], that OPA–amino drug adducts exhibit interesting electrooxidative properties in relation to their isoindolic structure. We have applied this fact to a sensitive amperometric detection in a HPLC system for assay of various drugs.

## EXPERIMENTAL

### *Chemicals*

All chemicals used were of analytical reagent grade. Solvents were distilled twice before use. *o*-Phthalaldehyde (OPA) was purchased from Fluka (Buchs, Switzerland), 2-mercaptoethanol (MERC), ethanethiol (ET) and *tert.*-butylmercaptan (t-Bu) from Aldrich(Beerse, Belgium).

The structures and sources of the amino drugs studied are given in Table I.

### *Macro-scale synthesis of OPA–amino drug adducts*

Equal amounts of molar solutions in 95% ethanol of OPA and t-Bu were mixed and cooled for 15 min in ice. This mixture was allowed to stand at room temperature for 10 min before an equal amount of molar ethanolic solution of the amino drug was added and this mixture was kept overnight at –20°C. The OPA–amino drug adducts were obtained as oil or crystals and were isolated.

### *Mass spectra*

The chemical structures of the synthetic products obtained above were confirmed by using a LKB 2091 mass spectrometer by direct introduction and operating it as follows: ion energy source, +70 eV; source temperature, 280°C; accelerating voltage, 3500 V; and "trap" current, 50 $\mu$A.

### *Voltamperometry*

A polarographic analyzer (Model 174 A; E. G. & G. Princeton Applied Re-

search, Princeton, NJ, U.S.A.) was used in conjunction with a classical three-electrodes cell. This system incorporated a rotating glassy carbon working electrode (Model EDI + CONTROVIT; Tacussel, Villeurbanne, France; disk diameter 3 mm; speed 600 rpm), a saturated calomel reference electrode (SCE) (Model C-10, Tacussel) and a platinum-wire auxiliary electrode.

Recordings in direct current (d.c.) differential pulse (D.P.) and cyclic modes were made at scan rates of 2, 5 and 200mV/sec, respectively. In D.P. voltammetry the pulse height was 10mV and the pulse repetition was 0.5 sec.

*Chromatographic conditions*

The HPLC system included a ternary solvent delivery pump (Model S.P. 8700; Spectra-Physics, Santa Clara, CA, U.S.A.), an injection valve with a 10-$\mu$l sample loop (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and an UV–visible detector (Model LC 871; Pye Unicam, Cambridge, U.K.).

For amperometric detection, a thin-layer electrolytic cell (Model LCC 231; Merck-Clevenot, Nogent/Marne, France), fitted with glassy carbon working and auxiliary electrodes and a SCE was used in connection with an electronic control unit (Model E 230 Merck-Clevenot).

Chromatographic recordings and all calculations were performed on an integrator (Model ICR-1; Intersmat, Courtry, France).

Reversed-phase columns were prepacked with LiChrosorb RP-18 (Hibar E. C. 250-4, particle size 5 $\mu$m; E. Merck). Pre-columns (30 $\times$ 4 mm I.D.), packed with LiChrosorb RP-18 (particle size 40 $\mu$m) were used in-line for all chromatographic analyses.

The mobile phase consisted of various aqueous 12.5 m*M* disodium hydrogen orthophosphate buffer (pH 7.2)–methanol mixtures, which were filtered through a 0.6-$\mu$m microfilter (Type BD; Millipore, Bedford, MA, U.S.A.). The flow-rate was 70 ml $h^{-1}$.

The spectrophotometric detector was operated at $\lambda$ = 340 nm and the amperometric detector at an applied potential of + 0.9 V *vs.* SCE.

*Derivatization procedures*

The OPA– and thiol–amino drug derivatives were prepared in methanol–borate buffer mixtures. Borate buffer (pH 9.5) was prepared by dissolving orthoboric acid in water (3.3%, w/v) and adjusting the pH to the correct value with 10 *M* sodium hydroxide solution.

For voltamperometry the test compounds were obtained by mixing 100 $\mu$l of $5.5 \cdot 10^{-1}$ *M* OPA, thiol and amino drug solutions in methanol with 100 $\mu$l of borate buffer. After waiting for 30 min, the resulting solution was added to 50 ml of the mobile phase used in the HPLC system. For HPLC $10^{-2}$ *M* stock solutions of standard drugs were prepared in methanol and then diluted in water. Reagents (OPA, MERC, ET, t-Bu) were dissolved at a concentration of $10^{-3}$ *M* in methanol and diluted before adding in a tenmolar excess to the drug.

Derivatization was performed by combining 100 $\mu$l of OPA solution, 200 $\mu$l of borate buffer and 500 $\mu$l methanol. After mixing, 100 $\mu$l of the amino drug solution ($10^{-4}$–$10^{-6}$ *M*) were added and the resulting mixture was allowed to react at room temperature in darkness for 30 min; an aliquot was injected into the liquid chromatograph.

## RESULTS AND DISCUSSION

### *Structural study*

Simons and Johnson[6,7] have reported structural data for the condensation products between OPA, thiol and *n*-propylamine in 1:1:1 molar ratio. The described structure was an additive ternary isoαindole-type product, which was generally accepted by other authors[2,10].

However, other minor compounds could be formed during condensation, particularly in the presence of excess of OPA reagent. Addition products of the initial ternary structure and another OPA molecule have recently been described[14]. MERC is generally presumed to give a different isoindolic compound by elimination of the thiol substituent and appearance of a keto group[7,10].

In order to confirm the structure of the main product obtained by condensation of OPA, thiol and the amino drugs, we carried out a macro-scale synthesis of these adducts under conditions close to those previously reported[6,7]; t-Bu was used as thiol compound because it allowed isolation of the corresponding adducts, in contrast to MERC and ET, which are usually employed for derivatization in HPLC analysis.

Mass spectral data, obtained from these synthetic adducts, confirm the ternary addition product. The molecular ion values were those expected for each sympatomimetic drug derivative (Table II). Also, the fragmentation scheme exhibits ions which result from the elimination of the thiol substituent (II, III and II', III') and next from removal of the substituent of the amino group (IV and V) (Fig. 1).

### *Electroactive properties*

d.c. voltammetry exhibited a well-defined wave ($E_{1/2}$ = 0.50 V) for the OPA–drug derivatives. The electroactivity of the reagents alone was tested. They produced a slight rise of the residual current level obtained with the mobile phase (Fig. 2). D.P. voltammetry showed a symmetrical peak with a maximum value at $E_p$ = 0.52 V, which is close to the $E_{1/2}$ value. Cyclic voltammetry exhibited oxidative and reductive waves with $E_{1/2}$ values at almost the same potential. This fact may indicate a rapid electron-exchange process.

The electroactivity of the OPA–amino drug adducts was tested at various pH values in phosphate buffers, ranging in pH from 3.0 to 7.2, which is commonly used

TABLE II

CHARACTERISTIC IONS OF OPA–AMINO DRUG ADDUCTS, OBTAINED BY MASS SPECTROMETRY (FIG. 1)

| *OPA amino drug adducts* | *m/z values* | | | |
|---|---|---|---|---|
| | *I* | *II* | *V* | *II'* |
| Norephedrine | 339 | 283 | 135 | 282 |
| Heptaminol | 333 | 277 | 129 | 276 |
| Amphetamine | 323 | 267 | 119 | 266 |
| Phenethylamine | 309 | 253 | 105 | 252 |
| 2-Heptylamine | 303 | 247 | 99 | 246 |

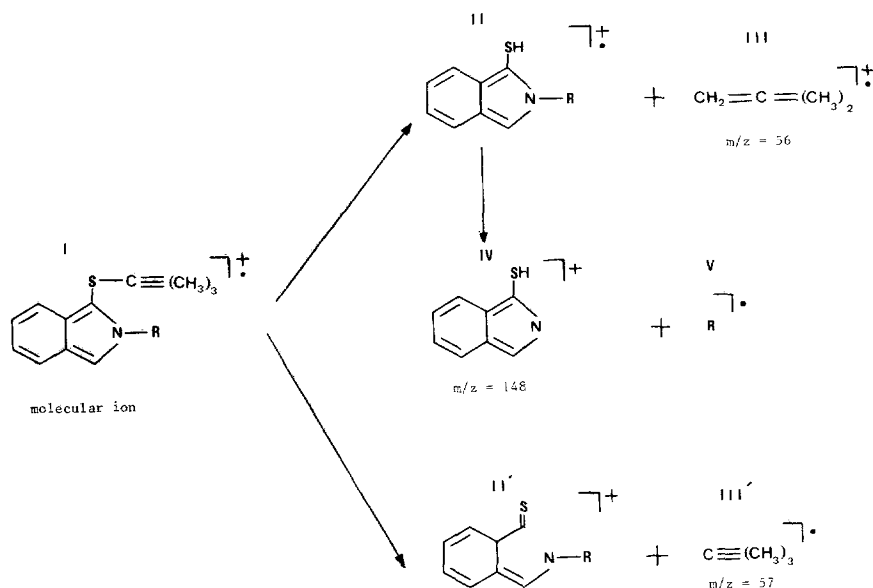


Fig. 1. General mass spectrometry fragmentation scheme of OPA-amino drug adducts.

in reversed-phase chromatography. No significant variations occurred in the diffusion current level. Also, the nature of the thiol compound and the amino drug did not affect the results. Therefore, it seems that the electroactivity is related only to the isoindolic structure. By way of comparison, serotonin and its structural analogues, which are well-known indolic compounds, are currently measured by HPLC with electrochemical detection[13]. Other molecules with a heterocyclic nitrogen atom have also been cited for their electroactive properties[15,16]. The unsaturated heterocycle resulting from the condensation of primary amino compounds with OPA produces a high density of electronic charge, which can give rise to an electron-exchange process.

*Chromatographic study*

The chromatography of OPA-amino compound adducts obtained by pre-column derivatization has been described for amino acids[9], biogenic amines[10] and sympatomimetic drugs [2,12] on silica, bonded with alkyl chains ($C_8$ or $C_{18}$), as stationary phase. Mobile phases used with these reversed-phase columns usually consist of methanol or acetonitrile-and aqueous buffer mixtures (pH 3–8).

We tested an acetate buffer (pH 3.0) and a phosphate buffer (pH 7.2) on a LiChrosorb RP-18 column. The latter afforded sharper peaks for OPA-drug adducts and consequently better resolution for neighbouring peaks than the former. Two ways of obtaining a different capacity ratio for each OPA–drug adduct were investigated. First, various proportions of methanol were added to the phosphate buffer. Then, different thiols (MERC, ET or t-Bu) were used in the derivatization procedure (Table III). It should be noted that the capacity ratio of an OPA–drug adduct increases with the thiol employed in the following order: MERC $<$ ET $<$ t-Bu. This

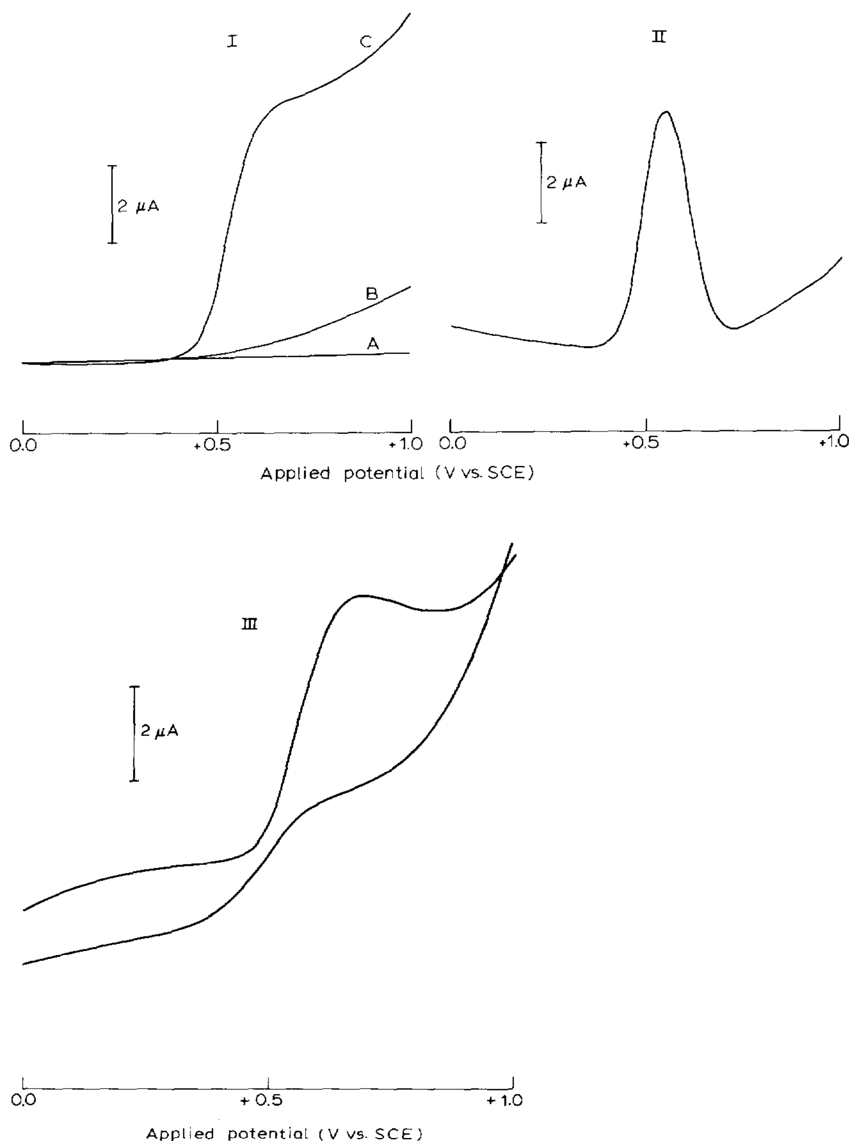


Fig. 2. d.c. (I), D. P. (II) and cyclic (III) voltamperograms of OPA–amino drug adducts. I was carried out in methanol–phosphate buffer, pH 7.2 (70:30, v/v) alone (A) with the addition of OPA, thiol and borate buffer (B) and with the addition of OPA, thiol, borate buffer and amino drug (C). II and III were obtained under the same conditions as IC.

fact demonstrates that the alkyl substituent of the thiol group stays on the isoindolic derivative during the chromatographic analysis.

The derivatization procedure was designed to decrease the excess of reagents, because they have electroactivity and exhibit peaks close to the injection dead-volume. A lower applied potential did nothing to minimize their intensity. A great excess

TABLE III

CAPACITY RATIOS, $k'$ OF OPA-AMINO DRUG DERIVATIVES, OBTAINED WITH VARIOUS THIOL COMPOUNDS

Methanol-phosphate buffer (pH 7.2) mixtures as mobile phase in the proportions: *65:35;**70:30; ***85:15.

| | *Amino drug* | | | | |
|---|---|---|---|---|---|
| *Thiols* | *Norephedrine* | *Heptaminol* | *Amphetamine* | *Phenethylamine* | *2-Heptylamine* |
| MERC | 1.93 * | 3.47* | 7.33* | 2.86** | 6.71** |
| ET | 9.46** | 12.43** | 30.26** | 4.17*** | 9.21*** |
| t-Bu | 2.72*** | 3.21*** | 7.00*** | 7.21*** | 13.6*** |

of reagent, injected into the HPLC system, upsets the electrochemical detector response so that it cannot be reset to the baseline before detection of the OPA-drug adducts.

Typical chromatograms are shown in Fig. 3.

Derivatization of sympatomimetic drugs with OPA and detection of the resulting adducts by UV spectrophotometry greatly increase the sensitivity of HPLC (average $\varepsilon_M$ 5000) value in comparison with the underivatized compounds. Moreover, the electrochemical detector permits detection at a level of 0.3 ng of injected drug, which is a better result than those previously obtained with fluorimetry (30 ng injected)[2]. The sensitivity of the reported method is also superior to that of RIA and EMIT assays[1].

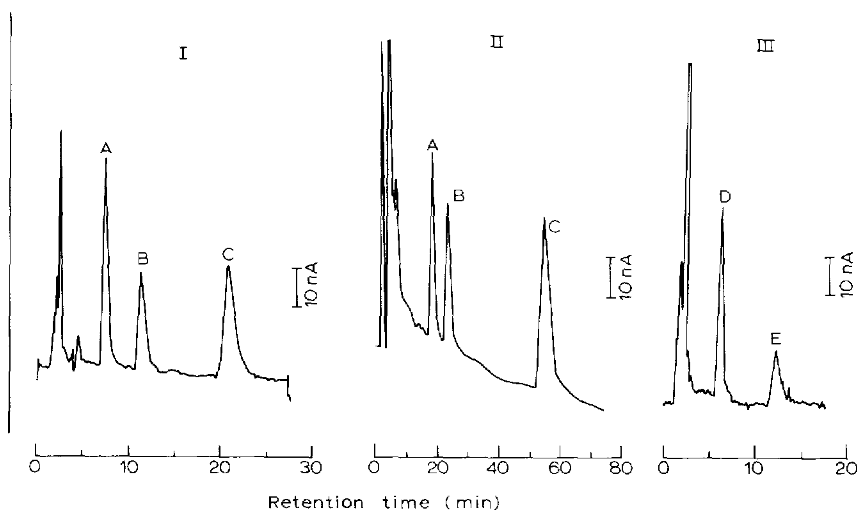


Fig. 3. Chromatograms of OPA-amino drug adducts on a LiChrosorb RP-18 column with methanol-phosphate buffer (pH 7.2) at a flow-rate of 70 ml $h^{-1}$ and with electrochemical detection at an applied potential of + 0.9 V. About $10^{-11}$ mole of each compound was injected. I Norephedrine (A), heptaminol (B) and amphetamine (C), derivatized with OPA and MERC, and chromatographed in methanol buffer (65:35); II as in I but ET and 70:30 solvent ratio; III phenethylamine (D) and 2-heptylamine (E), derivatized with OPA and MERC, and chromatographed with a 70:30 solvent ratio.

## CONCLUSIONS

So far no isoindolic compounds have been studied with regard to their electroactive character. The newly described electrooxidative property of OPA-amino compound adducts increases the number of available detection modes for these compounds. The UV spectrophotometric detection of these adducts affords sufficient sensitivity for the measurement of primary amino compounds in usual concentrations. Fluorimetric and electrochemical detections are well suited to the trace analysis of these compounds in biochemical, pharmacokinetic and environmental applications. HPLC systems with electrochemical detection are now being developed thanks to the low cost and improved performances of this equipment.

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