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Short Communication

Liquid chromatographic determination of a quaternary ammonium-steroidal type drug in plasma with post-column extraction

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Introduction

Post-column reaction detection is becoming increasingly important in column-liquid chromatography (LC). In cases where the solute has little or no ultraviolet-visible absorbance or fluorescence, post-column reactions can enhance selectivity and sensitivity. One of the techniques used is ion-pair extraction of the solute with a counter-ion which is strongly absorbing or fluorescent [1, 2]. The counter-ion is added directly to the mobile phase or is added after the chromatographic separation. After the separation the column effluent is segmented by an organic solvent and the ion-pair formed is extracted into the organic phase in a reaction coil. After desegmentation in a phase separator the organic phase is passed through the detector.

Compounds with tertiary amine or quaternary ammonium groups readily form ion-pairs with 9,10-dimethoxyanthracene-2-sulphonate (DAS), which is a strongly fluorescent compound [2, 3]. Org 7684 (1-[(2β,3α,5α,16β)-3-(acetyloxy)-2-(1-piperidinyl)-androstan-16-yl]-1-(2-propenyl)-piperidinium bromide) (Fig. 1) was selected as a model compound for non-depolarizing neuromuscular blocking agents of the Norcuron type [4]. Analogues of this compound are often separated by normal-phase LC and detected by UV absorbance measurements [5]. In the present communication a method is described for the separation and detection of Org 7684 and its hydrolysis product Org 7678 (Fig. 1) at the low nanogram level using LC with post-column extraction and fluorescence detection.

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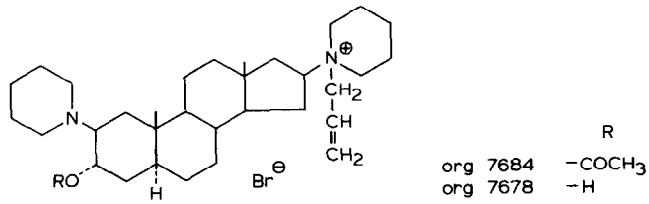

Figure 1
The structures of Org 7684 and its hydrolysis product Org 7678.

Materials and Methods

Reagents

Dioxane and dichloroethane were purchased from Baker (Deventer, The Netherlands) and were used without purification. 9,10-Dimethoxyanthracene-2-sulphonate (DAS) was obtained from Fluka (Buchs, Switzerland). As eluent, mixtures of dioxane and an aqueous 0.1 M sodium phosphate buffer (pH 3.0) containing 15 mg of DAS per litre was used. Fresh blood was collected in heparinized tubes; plasma was prepared by centrifugation at 3000 ***g*** and stored at −20°C.

LC system

A schematic of the appartus is shown in Fig. 2. The solvents were delivered by two Kontron (Zürich, Switzerland) Model LC 414 pumps with Kontron pulse dampers. Samples were injected by means of a Valco (Houston, USA) six-port valve. The 125 × 4.6 mm i.d. analytical column packed with 10 μm Spherisorb ODS-2 (Phase Separations, Queensferry, UK) was a gift from Kontron (Switzerland). A restrictor was placed between the pulse damper of the segmenting solvent and the T-piece. The organic phase was added to the eluent stream via a Valco T-piece with a 0.25 mm bore. The extraction took place in a stainless-steel capillary (1.50 m × 0.8 mm i.d. coil diameter, 40 mm). The design of the wetting-type phase separator was based on that of a similar type described previously [6]. One half of the separator is constructed of PTFE, and the other half of stainless steel. With this separator it is possible to obtain a purely organic phase. A detailed description of the design and characteristics of the phase separator will be published elsewhere. The ratio of the flow through the detector to that to waste was regulated by an SGE (Melbourne, Australia) MCV 50 microvalve. A Kontron Model SFM 23 fluorimetric (λ_{ex} = 383 nm, λ_{em} = 452 nm) detector was used. Chromatograms were recorded on a Kipp (Delft, The Netherlands) BD-8 recorder.

Analysis of plasma

Disposable extraction columns of 1 ml volume packed with 40-μm C_{18} silica were purchased from Baker (Deventer, The Netherlands). As an alternative, 150 mg of 10

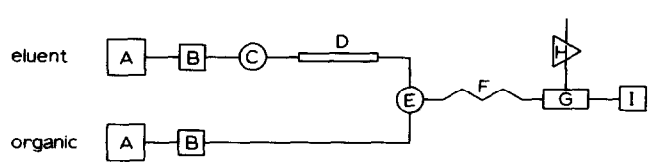

Figure 2
Schematic of the LC system. A, pump; B, pulse damper; C, injector; D, analytical column; E, T-piece; F, extraction coil; G, phase separator, H, needle valve, I, detector.

μm Spherisorb ODS-2 (Phase Separations) was used instead of the Baker C_{18} material. The extraction columns were pre-conditioned as recommended by the suppliers. Fresh plasma (2.0 ml) to which 300 μl of sodium dihydrogen phosphate [5] was added, was applied to the column as a blank. The column was first washed with 1 ml of a 100 mM phosphate buffer (pH 6) and then with 1.5 ml of a 100 mM sodium phosphate buffer (pH 3). The column was allowed to run dry and was eluted with 500 μl of mobile phase. Of the eluate, 100 μl was injected onto the analytical column. A mobile phase of 20% dioxane in 50 mM sodium phosphate buffer (pH 3) containing 15 mg DAS per litre was used. After chromatographic separation the eluent was segmented with dichloroethane. Both flow rates were 1 ml min^{-1}.

Results and Discussion

Chromatographic system

Preliminary experiments showed that the use of aqueous dioxane as eluent in combination with 10 μm Zorbax Cyano or Spherisorb ODS-2 columns gave the best results. With other types of columns, such as LiChrosorb RP-18, diol and other cyano columns, no elution of the analytes Org 7684 and Org 7678 from the column was observed. Substitution of methanol or acetonitrile for dioxane did not result in satisfactory elution patterns with any of the columns tested. The separation of Org 7684 and Org 7678 on the Spherisorb ODS-2 column with 20% aqueous dioxane as modifier is shown in Fig. 3.

As regards phase separation, passing 30–40% of the dichloroethane flow to the detector cell was found to be a good compromise in terms of band broadening and sensitivity. Lawrence *et al.* [2] showed that the noise of their post-column reaction system increased with an increasing proportion of modifier in the LC effluent. The background signal also showed a steep increase because of co-extraction of DAS into the organic phase. As revealed in Fig. 4, this is not the case in the present dioxane-based system. At 10% of dioxane the background fluorescence of the dichloroethane phase reached a maximum.

Upon prolonged use the retention increases and the column performance decreases. Both can be readjusted by increasing the dioxane concentration from 20 to 30%.

Plasma samples

Plasma samples cannot be applied directly onto the separation column because of their high protein content. The clean-up procedure described by Paanakker and Van de Laar [5] for Org NC 45 does not yield satisfactory results for Org 7684 and Org 7678. In that procedure the compound is extracted as an ion-pair into an organic phase, the solvent is evaporated and the residue redissolved in the eluent.

Keeping automated on-line preconcentration in mind [7], a sample preparation procedure using disposable extraction columns was developed. When using a C_{18} column a recovery of about 80% was achieved at concentrations of 1 μg per ml plasma. On decreasing the amount applied onto the column the recovery is found to decrease: at concentrations of 100 ng per ml plasma the recovery was 50–60% and at concentrations of 30–50 ng per ml plasma, subsequent elution with three portions of 500 μl of eluent each resulted in recoveries of 30, 18 and 12%, respectively. These results suggest irreversible binding, probably caused by residual silanol groups. Replacing the Baker C_{18} disposable column by 150 mg of Spherisorb ODS-2 in the same precolumn eliminated

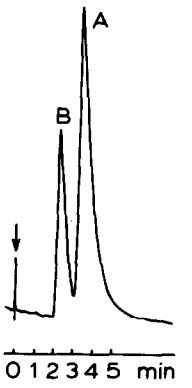

Figure 3
Separation of Org 7684 (A) and Org 7678 (B). Chromatographic conditions: Column, 10-μm Spherisorb ODS-2; mobile phase, 20% dioxane in 50 mM sodium phosphate buffer (pH 3) containing 15 mg/l DAS (1 ml min^{-1}). Organic phase, dichloroethane (1 ml min^{-1}); flow of dichloroethane through detector, 0.35 ml min^{-1}.

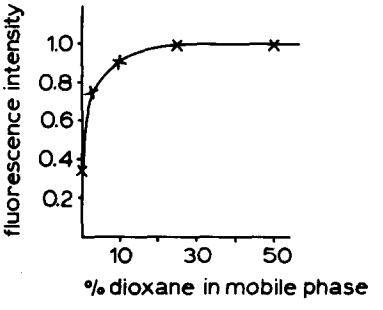

Figure 4
Dependence of fluorescence intensity of dichloroethane phase on percentage of modifier in the eluent.

this problem. Up to 100 ng of Org 7684 per ml plasma the recovery now is close to 100%. However, at concentrations higher than 100 ng per ml plasma the recovery decreased; for example, 300 ng per ml plasma gave a recovery of 60%. With 250 mg of Spherisorb ODS-2 the recovery increased to 80% for the same 300 ng per ml plasma sample, indicating a lower capacity of the Spherisorb ODS-2 material.

The detection limit (signal to noise ratio of 3) of Org 7684 in plasma is the same as that in standard solutions, *viz.* 2.5 ng, as shown in Figs 5A and 5B. The calibration curve was linear ($r = 0.9989$) over at least three orders of magnitude. At a concentration level of 30 ng of Org 7684 per ml plasma, the repeatability of the method was better than ±3% (rel. S.D. at $n = 4$). During the analyses, no decomposition of the drug was observed.

Conclusion

A reversed-phase LC system combined with a post-column ion-pair reactor and a fluorescence detector has been used to separate the quaternary amino-steroidal drug Org 7684 and its hydrolysis product Org 7678, and to determine Org 7684 quantitatively at the low nanogram level.

For adequate sensitivity and selectivity, off-line preconcentration and sample clean-up with a short precolumn is required. For concentrations up to 300 ng per ml plasma the use of Spherisorb ODS-2 is recommended as the packing material of the precolumn. At higher concentrations the ODS-2 should be replaced by Baker C_{18}.

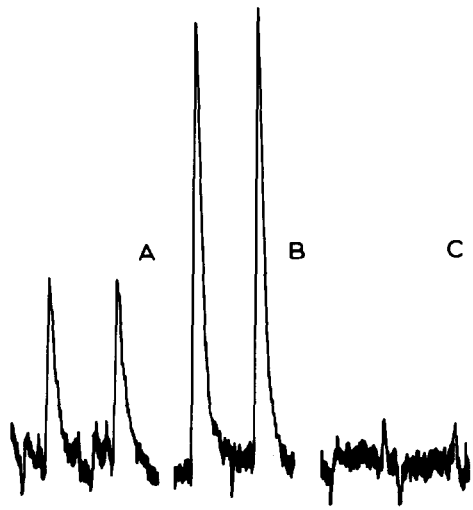

Figure 5
(A) Duplicate LC analyses of plasma sample spiked with 8.5 ng ml^{-1} of Org 7684. Plasma (2 ml) is applied onto an extraction column packed with 150 mg of 10 μm Spherisorb ODS-2. The analyte is eluted with 500 μl of eluent, 100 μl of which are injected (recovery, 97%). (B) Duplicate direct injection of 8.5 ng of Org 7684. (C) Blank plasma, procedure as in Fig. 5A. Chromatographic conditions as shown in Fig. 3.

The detection principle based on DAS ion-pairing can conceivably be extended to other amino-steroidal drugs containing a quaternary ammonium group.

The routine use of this technique for large series of samples and the automation potential via column-switching is currently under investigation.

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