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On-line phase-transfer catalysed dansylation of phenolic compounds followed by normal-phase liquid chromatography with fluorescence detection

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Abstract: A study of the on-line phase-transfer catalysed dansylation of phenolic compounds is presented. The extraction–dansylation is performed in the extraction coil of a home-made flow-injection extraction unit. After phase separation, the organic phase is fed to a normal-phase liquid chromatographic system with fluorescence detection. Ethynyloestradiol, oestradiol and paracetamol are used as test compounds. The influence of temperature on the reaction is examined. Calibration graphs showed good linearity ($r > 0.996$) and limits of detection are satisfactory (8×10^{-7} M for ethynyloestradiol, 2×10^{-6} M for oestradiol and 5×10^{-7} M for paracetamol). The method is not applicable for the assay of oxychinoline, phenylephrine and morphine.

Keywords: *Phase-transfer catalysis; on-line dansylation; ethynyloestradiol; oestradiol; paracetamol; normal-phase liquid chromatography; fluorescence detection.*

Introduction

Dansyl chloride (DAC: 5-(dimethylamino)-1-naphthalenesulphonyl chloride) is used widely as a fluorescence labelling agent for primary and secondary amines and phenols [1, 2]. Usually, the dansylation is performed at room temperature in an acetone–water mixture the pH of which is adjusted by a suitable base (e.g. sodium carbonate). Reaction times are of the order of 30 min [3]. However, under these conditions several side reactions may occur [2].

The dansylation of phenolic compounds can be accelerated significantly by a phase-transfer catalysed procedure, as described by De Ruiter *et al.* [4]. They demonstrated that dansylation of phenolic steroids (ethynyloestradiol and oestradiol) at room temperature, using phase-transfer catalysis, is fast enough to be applied as an on-line procedure combined with liquid chromatography (LC) [5]. The phenolic compounds are deprotonated in alkaline aqueous medium (1.0 M NaOH), and subsequently extracted as an ion pair with tetrabutylammonium (TBA) ions into the DAC-containing organic phase, in which the dansylation takes place. In addition to the dansylated analyte, a major side product was observed, which was claimed to be the dansyl dimer, but no structural evidence was given. The proposed reaction scheme is shown in Fig. 1 [4].

The phase-transfer catalysed dansylation can be performed both pre- and post-column [5]. In the pre-column mode, the clean-up properties of the extraction step are fully utilized; this is particularly favourable when complex matrices have to be analysed (e.g. urine). To automate the dansylation in the pre-column mode, De Ruiter *et al.* [5] performed the process of dansylation in vials of an autosampler, by filling these with the aqueous sample and, subsequently, adding the aqueous reagent (TBA ions in 1.0 M NaOH) and the organic reagent (DAC in methylene chloride).

In this paper the feasibility of pre-column extraction–dansylation with an on-line flow-extraction unit is explored. This approach combines the advantage of the pre-column clean-up step with a more flexible control of the reaction conditions (e.g. temperature) leading to improved sample through-put and detection limits. Normal-phase LC is used to separate the dansylated compounds. The applicability of the method to paracetamol and some other pharmaceutically relevant phenolic compounds is demonstrated.

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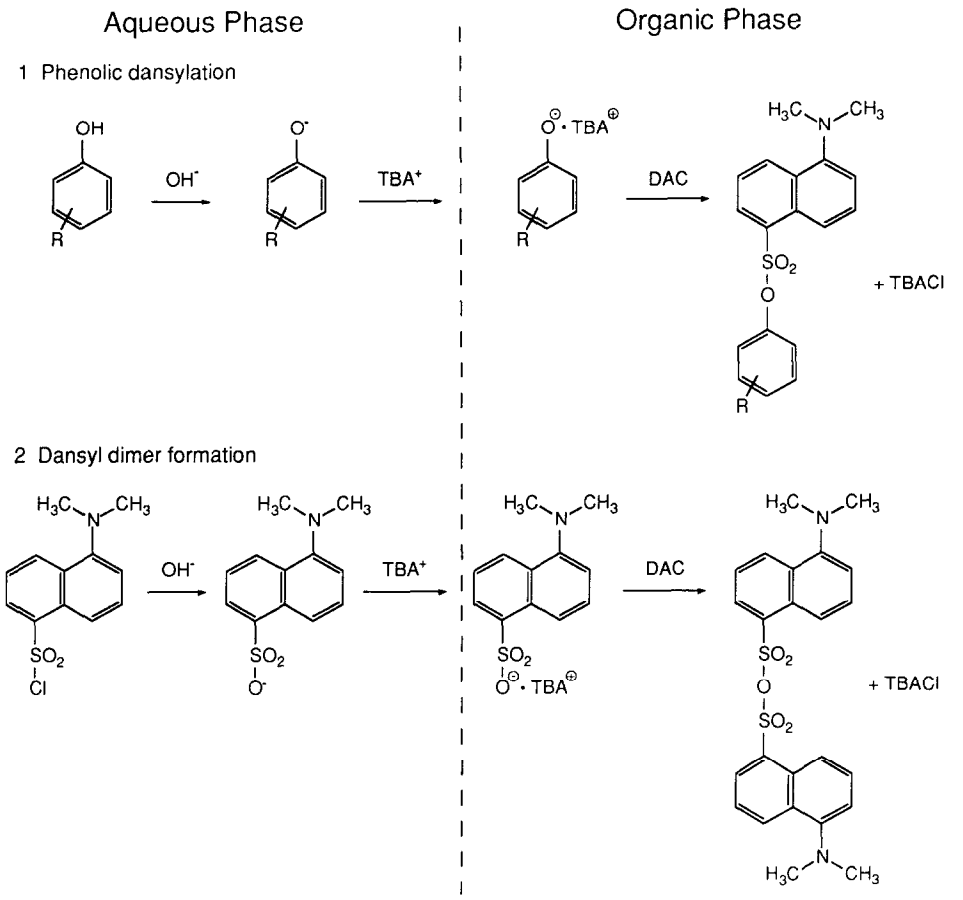

Figure 1
Proposed reaction scheme of the phase-transfer catalysed (1) dansylation of phenolic compounds and (2) dimerization of dansyl chloride.

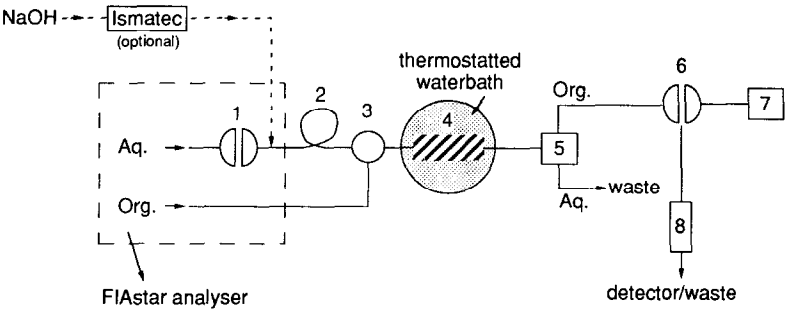

Figure 2
Schematic representation of the on-line dansylation–LC system, using phase-transfer catalysis. (1) Injector of the extraction unit (200-μl loop); (2) mixing coil (optional); (3) segmentor; (4) extraction coil; (5) separator; (6) injector of the LC unit (100-μl loop); (7) LC pump; (8) analytical column.

Experimental

Chemicals and solutions

Aqueous solutions were prepared with doubly distilled water. Chloroform and methylene chloride were of p.a. quality (Merck, Darmstadt, Germany). Acetonitrile (HPLC quality) was obtained from Westburg (Leusden, The Netherlands) and *n*-hexane (p.a. quality) from Janssen Chimica (Geel, Belgium). Tetra-*n*-butylammonium salts (Fluka Chemie, Buchs, Switzerland) were of purum (bromide and hydroxide) and puriss. p.a. (iodide) quality. Dansyl chloride (puriss.

p.a.) was from Fluka. Ethynyloestradiol and paracetamol (all Ph. Eur. quality) were obtained from OPG Farma (Utrecht, The Netherlands). All chemicals were used as received.

Aqueous stock solutions (approximately 10^{-3} M) of the analytes were prepared daily in 0.01 M TBA, with or without 0.5 M NaOH. For calibration, these solutions were diluted with a NaOH–TBA or TBA solution. The urine samples were spiked with ethynyloestradiol and subsequently diluted 1:1 with the NaOH–TBA solution.

Apparatus and methods

The experimental set-up is depicted schematically in Fig. 2. The organic and aqueous phases of the extraction unit were pumped by a FIAstar 5020 analyser (Tecator, Höganäs, Sweden), using tygon (Bran and Luebbe, Maarssen, The Netherlands) pump tubes and a displacement bottle for the organic phase. The injector of the FIAstar analyser, provided with a 200-µl loop, was used to inject the sample into the carrier (doubly distilled water).

The carrier was segmented with the organic phase (a solution of DAC in either chloroform or methylene chloride) in an Omnifit (Cambridge, UK) PTFE T-piece. The extraction–dansylation took place in an extraction coil of variable length (0.5 mm i.d. PTFE tubing). This coil (coiling diameter 1 cm) was placed in a thermostatted water bath. After extraction, the phases were separated with a Tecator coiled groove membrane separator, mounted in a home-built unit consisting of two stainless-steel disks equipped with Omnifit fittings; the disks were fastened together with three nuts and bolts. The properties and dimensions of the separator have been described previously by Sahleström and Karlberg [6]. After separation, the organic phase was fed into the 100-µl loop of the LC-unit injector (model N60, Valco, Houston, TX, USA). The time needed by the sample plug to arrive at the LC-injector was visually determined by injecting a sample with a high concentration of both TBA and picrate. The time required was approximately 1.5 min (depending upon the extraction coil length and the flow rates used).

The mobile phase of the LC unit was pumped by a M6000 LC pump (Waters, Milford, MA, USA). Separation took place at ambient temperature on a 30 cm × 3.9 mm i.d. column, packed with LiChrosorb SI 60, 10 µm (Merck). The LC-unit further consisted of a Spectroflow 980 fluorescence detector (Kratos, Ramsey, NJ, USA), with excitation and emission wavelengths set at 340 and 420 nm, respectively.

Typical flow rates were 0.5 ml min^{-1}, for both the aqueous carrier and the organic phase of the extraction unit, and 1.0 ml min^{-1} for the mobile phase of the LC-unit.

The high pH required for deprotonation of the phenolic moiety may induce decomposition of some analytes. In order to reduce the time the analytes are exposed to this pH, the extraction unit was provided with the option to add NaOH on-line to the injected sample, which could in this case be a solution of analyte in 0.01 M TBA, without NaOH. In this option, an IPN-8 peristaltic pump (Ismatec, Zürich, Switzerland), with variable pump speed and equipped with silicone pump tubes (Bran and Luebbe), delivered the NaOH stream to the extraction unit. The injected sample and the NaOH stream were allowed to mix in a mixing coil, placed before the segmentor. To keep the flow rates and ratios constant, adjustment of the pH was achieved by feeding various NaOH concentrations to the Ismatec pump, instead of varying the flow rate delivered by this pump. The flow rate was set at 0.1 ml min^{-1}.

Results and Discussion

General features/ethynyloestradiol

Commonly used pump tubes are incompatible with the high pH needed for deprotonation of phenolic compounds. Pump tubes that are compatible are much more expensive. Therefore, in order to reduce costs, it was decided to investigate whether it is possible to use pure water as the carrier for the extraction unit while adjusting the pH of the samples prior to injection. This required that a rather high volume (200 µl) of the analyte had to be injected, in order to ensure that the pH was constant in the major part of the sample plug during transport and extraction. Only when results were obtained that indicated instability of the analyte at high pH values was the option of on-line addition of NaOH (see Experimental) investigated.

Ethynyloestradiol was chosen as the first test compound. The influence of the extraction-coil length at room temperature was investigated, with methylene chloride as the organic phase

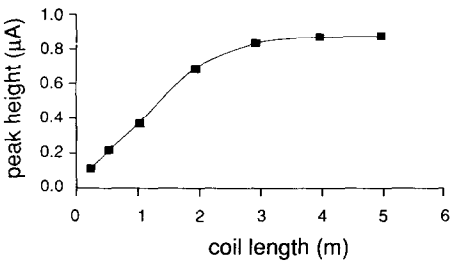

Figure 3
Influence of the length of the extraction coil on the on-line dansylation of ethynyloestradiol. Analyte: 10^{-5} M ethynyloestradiol in 0.5 M NaOH and 7.5×10^{-3} M TBABr. Conditions: organic phase extraction unit: 2.8×10^{-4} M DAC in methylene chloride; mobile phase LC unit: chloroform–hexane (80:20, v/v); experiments were performed at ambient temperature.

for the extraction unit. Too short coils may result in both incomplete extraction and dansylation. The shortest possible connection between the phase segmentor and separator was 5 cm. No significant improvement in peak heights was observed for coils longer than 3 m (Fig. 3). Consequently, for subsequent experiments with ethynyloestradiol the 3-m coil was employed.

Two peaks were always observed; the first, eluting near t_0, also was present when blank solutions (i.e. a solution of 0.5 M NaOH and 0.01 M TBA) were injected, and was attributed to the compound that De Ruiter *et al.* [4] claimed to be the dansyl dimer. The retention of this compound indicates a less polar nature than dansylated enthynyloestradiol which possesses a hydroxyl group which may increase retention. The suggested dansyl dimer lacks such a polar group. Because the compound could be separated from dansylated ethynyloestradiol and did not interfere in the assay, no further attempts to elucidate the identity of the compound were taken.

Normal-phase LC systems are extremely sensitive to the presence of water in the mobile phase. Because the organic phase of the extraction unit is saturated with water it was decided in the preliminary experiments to add water to the mobile phase as a standard routine. However, after several injections a decrease of the height of the dansylated ethynyloestradiol peak was observed, whereas the peak near t_0 increased in size. This was not the case when a water-free mobile phase was used. These phenomena are not fully understood, although it seems probable that the decrease of the dansylated ethynyloestradiol peak is caused either by difference in solvent polarity or by on-column hydrolysis. Therefore, water-free mobile phase was used throughout all further experiments.

The influence of the nature and the concentration of the counter ion of the TBA salts on the extraction–dansylation of ethynyloestradiol also was investigated. Figure 4 shows the results and indicates that the nature of the counter ion plays an important role in the overall process. A possible explanation is the variation in extractability of the counter ion as ion pair with the TBA ions. The larger the counter ion, the higher the extractability, and the lower the amount of TBA available in the aqueous phase for interaction with deprotonated ethynyloestradiol. Dissociation of the TBA ion pair in the organic phase could further reduce the concentration of TBA ions in the aqueous phase. Again, dissociation would be expected to be more pronounced for the larger counter ions.

In a comparable experiment by De Ruiter *et al.* (peak height vs reaction time for different TBA salts) the order $OH^- > Br^- > Cl^- > I^-$ was observed [5]. The final concentration of dansylated ethynyloestradiol was the same for all counter ions [5]. Surprisingly, chloride is situated between bromide and iodide, when one would expect the sequence to be $Cl^- > Br^- > I^-$. However, because TBACl is formed in the organic phase during dansylation, the overall reaction rate decreases due to saturation of the organic phase with this ion pair.

From Fig. 4 it was concluded that the optimal extraction coil length also depends on the nature of the counter ion (the shortest coils can be used when the counter ion is OH^-).

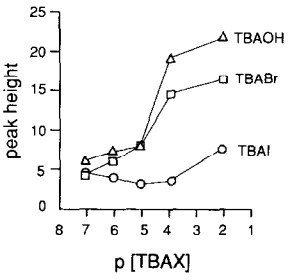

Figure 4
Influence of the TBA counter-ions on the on-line dansylation of ethynyloestradiol. Analyte: 10^{-7} M ethynyloestradiol in 0.5 M NaOH and various concentrations of TBAX. Conditions: organic phase extraction unit: 2.8×10^{-4} M DAC in methylene chloride; mobile phase LC unit: chloroform–hexane (70:30, v/v); length of extraction coil: 3 m; experiments were performed at ambient temperature.

In blank solutions (i.e. solutions of TBA salts in 0.5 M NaOH) a compound was present that coeluted with dansylated ethynyloestradiol in amounts equivalent to approximately 8×10^{-8} M ethynyloestradiol. The use of solutions made with doubly distilled water instead of demineralized water resulted in an approximately 10-fold decrease of this blank peak. The blank peaks were of equal height for both TBABr and TBAI, but for TBAOH the peak was approximately four times higher. Attempts to separate the interfering and dansylated ethynyloestradiol peaks with various mobile phases were unsuccessful; the presence of this compound therefore determines the accuracy of the ethynyloestradiol assay. Assuming an interferent peak height equivalent to 8×10^{-9} M ethynyloestradiol (using doubly distilled water), and accepting 2% contribution of the interfering compound to the ethynyloestradiol peak, the calculated minimum detectable concentration is 4×10^{-7} M. A calibration graph for ethynyloestradiol shows good linearity over two orders of magnitude (Table 1).

Spiked urine samples also were investigated, an example is shown in Fig. 5. In the blank urine sample a substance coeluted with ethynyloestradiol. The interfering peak is higher than in the standard aqueous solutions. The calculated limit of determination, based on a maximum contribution of the interfering compound of 2%, is 8.0×10^{-7} M. No alternative mobile phases were tested.

No difference in peak heights was observed when freshly prepared stock solutions were compared with solutions that were several days old. Ethynyloestradiol seems therefore to be stable in the 0.5 M NaOH solutions; therefore, the option of on-line addition of NaOH was not investigated.

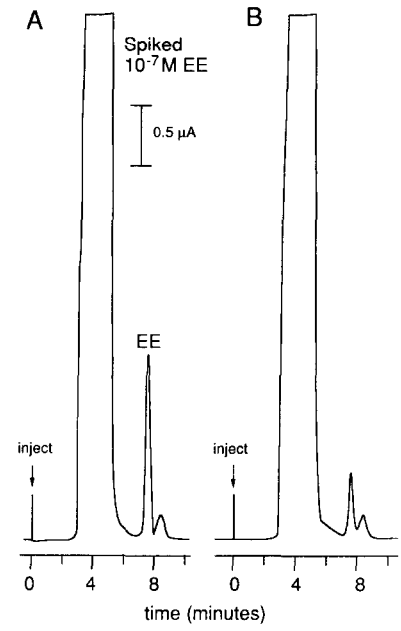

Figure 5
Assay of ethynyloestradiol in urine samples (A) spiked with 1.0×10^{-7} M ethynyloestradiol and (B) blank. Conditions: organic phase extraction unit: 2.8×10^{-4} M DAC in methylene chloride; mobile phase LC unit: chloroform–hexane (70:30, v/v); length of extraction coil: 3 m; experiments were performed at ambient temperature.

At the end of each working day the analytical column was flushed with methanol; it appeared that a large amount of fluorescent material had collected on the column during experimentation.

Oestradiol

For the experiments with oestradiol, chloroform instead of methylene chloride was used in

Table 1
Analytical data for the dansylation of various phenols

Compound	Concentration range (M)	Correlation coefficient	RSD (%)	LOD* (M)	Samples/hour
Ethynyloestradiol†	1×10^{-8}–1×10^{-6}	0.9992	2.1‡	8×10^{-7}	9
Oestradiol§	1×10^{-7}–5×10^{-5}	0.996	5.0‖	2×10^{-6}	9
Paracetamol¶	5×10^{-7}–1×10^{-4}	0.9994	2.5**	5×10^{-7}	8

*Limit of determination.
†Conditions: organic phase extraction unit: 2.8×10^{-4} M DAC in methylene chloride; sample dissolved in 0.01 M TBAI in 0.5 M NaOH; mobile phase: chloroform–hexane (70:30, v/v); extraction coil: 3 m; ambient temperature.
‡Three injections of 1.0×10^{-7} M.
§Conditions: organic phase extraction unit: 2.8×10^{-4} M DAC in chloroform; sample dissolved in: 0.01 M TBABr in 0.5 M NaOH; mobile phase:chloroform: hexane (70:30, v/v); extraction coil: 3 m, ambient temperature.
‖Seven injections of 1.0×10^{-7} M.
¶Conditions: organic phase extraction unit: 2.8×10^{-4} M DAC in chloroform; sample dissolved in: 0.01 M TBABr in 0.5 M NaOH; mobile phase: chloroform:acetonitrile (70:30, v/v); temperature water bath: 54°C; extraction coil: 5 m.
**Five injections of 1.0×10^{-5} M.

the extraction unit to enable the extraction–dansylation be be performed at elevated temperatures (boiling points of chloroform and methylene chloride: 62 and 40°C, respectively). However, raising the temperature of the water bath from 20 to 50°C reduced the peak heights by 32%. This was not further investigated, and further experiments were performed at ambient temperature.

For all mobile phases tested, dansylated oestradiol eluted at the same time as dansylated ethynyloestradiol. This implies that the accuracy of the oestradiol assay is also governed by the interfering peak, which was, under these conditions, approximately five times higher than in the case of ethynyloestradiol. This difference may be due to the different extraction solvents used. In the other analytical aspects ethynyloestradiol and oestradiol showed similar behaviour (Table 1), although the RSD for oestradiol was higher. The calculated limit of determination, accepting 2% contribution of the interfering compound, is 2×10^{-6} M. On-line addition of NaOH was not investigated, because there was no indication that oestradiol was unstable at the investigated pHs.

Paracetamol

Paracetamol was used as third test compound. Preliminary investigations showed that with a 2-m extraction coil and at room temperature no significant dansylation and/or extraction took place. Therefore, the experiments were repeated at elevated temperatures, with chloroform as the organic phase. The dependence of peak height on both temperature (up to 54°C) and extraction coil length (up to 5 m) were investigated. Neither a temperature nor a length optimum were observed. Further raising of the temperature is limited by the boiling point of chloroform. With a 5-m extraction coil and at 54°C the limit of determination for paracetamol was 5×10^{-7} M. A calibration graph for paracetamol is linear over two orders of magnitude (Table 1). The compounds that yielded peaks in the assay of blank solutions were separated from dansylated paracetamol.

When 2-day old solutions of paracetamol in 0.5 M NaOH and 0.01 M TBA were analysed, the paracetamol peak was lower (compared to a fresh solution) and a small, new peak appeared. This peak was attributed to *p*-aminophenol, originating from the hydrolysis of paracetamol. This assumption agrees with the results of injections of standard *p*-aminophenol solutions, because *p*-aminophenol has the same retention as the new peak. Fairbrother [7] has reported half-life times for paracetamol at various pH levels: 22 years at pH 6 and 2.3 years at pH 9 (both at 25°C). Although no information at higher pH is given, it is not unreasonable to assume that some *p*-aminophenol will be formed at pH >13 in a relatively short time.

Therefore, for paracetamol the option of on-line addition of NaOH to the injected sample plug was investigated. Standard solutions of paracetamol in 0.01 M TBA with and without NaOH were compared. It appeared that for both approaches the peak height increased with the pH of the NaOH solution that was added on-line. No increase of peak height was observed when longer mixing coils were used, indicating sufficient mixing in the original 25-cm coil. The highest NaOH concentration investigated was 2 M; there was no indication that maximum peak height was obtained, but the reproducibility at this NaOH level was less than at lower levels (Table 2). Peak heights of old alkaline paracetamol solutions were usually higher than of neutral ones, the difference depending on the age of the former solutions (a 2-day old alkaline solution still gave higher peaks than a neutral one with 1 M NaOH added on-line). In Fig. 6 the difference between an alkaline and a neutral solution is shown.

Table 2
Comparison of alkaline and neutral 10^{-3} M paracetamol solutions; on-line addition of NaOH

Added on-line [NaOH]	2 M	1 M	1 M
Type of sample	neutral	neutral	alkaline (fresh)
No. of injections	6	5	5
Mean peak height (mm)	163	136	206
RSD (%)	5.1	1.2	1.3

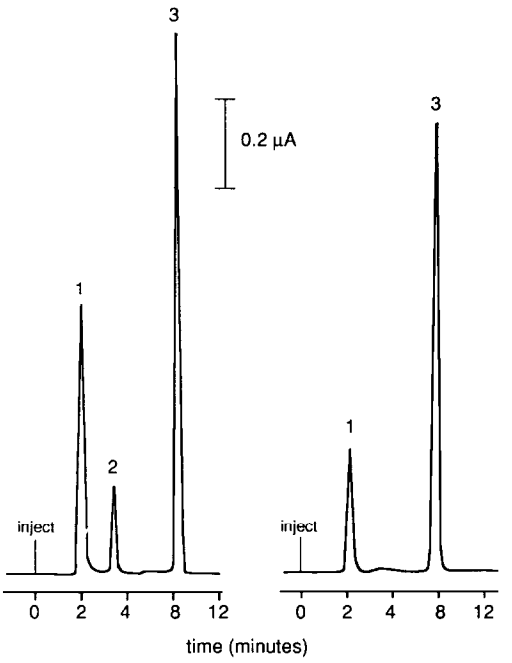

Figure 6
Assay of 10^{-3} M paracetamol. Left: 2-day old solution of 10^{-3} M paracetamol in 0.5 M NaOH and 0.01 M TBABr. Right: several days old solution of 10^{-3} M paracetamol in 0.01 M TBABr. (1) Claimed dansyl dimer; (2) dansylated *p*-aminophenol; (3) dansylated paracetamol. Conditions: organic phase extraction unit: 2.8×10^{-4} M DAC in chloroform; mobile phase LC unit: chloroform–hexane–MeOH (80:20:2, v/v/v); temperature water bath: 54°C; extraction coil: 5 m.

From these results it was concluded that on-line addition of NaOH is preferred for the assay of paracetamol. Although smaller peaks are obtained, the paracetamol content of neutral standard solutions remains constant over a longer period of time, thus increasing the reliability of the assay.

Other phenolic substances

Morphine, phenylephrine and oxychinoline were also tested at various conditions (temperature, length of extraction coil, extraction solvent, on-line addition of NaOH, chromatographic conditions, etc.) but in no case satisfactory results were obtained with the developed method.

Conclusions

Performing the dansylation on-line with a flow-injection extraction unit proved to be possible and improved the sample throughput (3–8 samples per hour reported [2] vs 8–9 in the present method). A major advantage of the method is the control of the reaction temperature, as illustrated for the assay of paracetamol. This compound was unstable at the high pH required, but hydrolysis could be satisfactorily reduced by on-line addition of NaOH, as opposed to using 0.5 M NaOH stock solutions.

For ethynyloestradiol, oestradiol and paracetamol, the method showed good linearity and the determination limits were satisfactory. The detection limits are not only dependent on the dansylation kinetics and the distribution coefficient of the analyte, but also on the purity of the chemicals used. By incorporating an autosampler, the method may be automated and applied to routine analysis.

The phase-transfer catalysed dansylation is not universally applicable to the assay of phenolic compounds. However, performing phase-transfer catalysis on-line in the flow-injection extraction mode by itself appeared to be fruitful and may be considered to be a useful application of flow-injection systems.

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