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SIMPLE EXTRACTION DETECTOR FOR THE LIQUID CHROMATOGRAPHIC DETERMINATION OF SECOVERINE IN BIOLOGICAL SAMPLES

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SUMMARY

A simple detection system for liquid chromatography based on post-column ion-pair extraction in a solvent-segmented stream is described. The fluorescent ion-pairing reagent 9,10-dimethoxyanthracene-2-sulphonate is added to the aqueous mobile phase prior to the column. An organic solvent, used for extraction of the ion pairs and for segmentation purposes, is added to the column effluent by gravity; about 70% of this phase is drawn through the detector cell using the siphon principle. The noise is significantly less than that of a system with a pulsating peristaltic pump. The system has been used for the detection of the prospective drug secoverine. Under optimized conditions, band broadening due to the extraction detection system is $\sigma_t = 7$ sec. The detection limit of secoverine is *ca.* 20 pg, the repeatability in the nanogram range is 2% (relative standard deviation) ($n = 6$) and the linear range is at least 4 decades. The use of a pre-column containing a 1–2-mm layer of stationary phase for clean-up and trace enrichment allows the direct injection of 200–1000 μl of plasma or serum. In this way, secoverine can be determined in the parts per 10^{12} range.

INTRODUCTION

The potential of extraction detection systems using air segmentation[1–3] and solvent segmentation[4–6] for the high-performance liquid chromatographic (HPLC) determination of amines has recently been demonstrated. Continuous extraction with solvent segmentation has also been used successfully in the flow-injection analysis of caffeine[7], codeine[8], vitamin B_1 [9] and anionic surfactants[10]. The solvent-segmentation principle combines the advantages of efficient suppression of band broadening and the removal of an excess of interfering reagent. Solvent segmentation was also found to be very useful for reactions with relatively slow kinetics such as the dansylation of primary and secondary amines[11].

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In this study, the segmentation principle was used for the detection of the prospective gastro-intestinal spasmolytic drug secoverine, using 9,10-dimethoxyanthracene-2-sulphonate (DAS)[12] as the fluorescent ion-pairing reagent. The design of the extraction detector was improved and simplified. In addition, the HPLC system was provided with a pre-column for trace-enrichment and clean-up purposes.

EXPERIMENTAL

Reagents

Fig. 1 depicts the structure of secoverine (Philips-Duphar, Weesp, The Netherlands), which is now under clinical investigation. A stock solution of the hydrochloride salt of secoverine was prepared in distilled water at a concentration of 100 mg/l. Dilutions of this solution were made as required. The sodium salt of the counter ion, DAS (Sandoz, Basle, Switzerland), was dissolved in distilled water at concentrations of 10^{-5}–10^{-4} *M*. All other chemicals were of analytical-reagent grade.

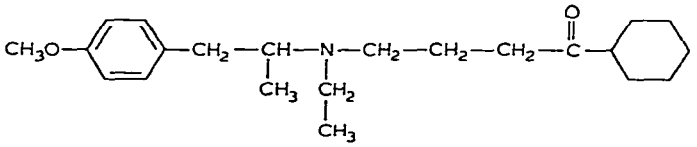

Fig. 1. Structure of secoverine.

High-performance liquid chromatography

The HPLC pump was an Orlita DMP-AE-10.4 pump (Orlita, Giessen, G.F.R.); the injection port was a Rheodyne six-port valve (Rheodyne, Berkeley, CA, U.S.A.) with a 200-μl loop. The analytical column was a stainless-steel tube of 20 cm × 4.6 mm I.D., packed by a slurry technique with CN-bonded 5-μm silica (HPLC-Sorb SIL 60-D 5 CN, Macherey, Nagel & Co., Düren, G.F.R.). For on-line/off-line switching, another Rheodyne six-port valve was inserted between the pre-column and the analytical column. The mobile phases are described below.

For the injection of plasma or serum samples, a pre-column[13] was used, consisting of a stainless-steel tube of 5 cm × 4.6 mm I.D. packed with a dense slurry of the same CN-bonded 5-μm silica in methanol using a micro-spatula. The length of the packing was 1–2 mm; the remaining part of the column was filled with a 2-μm frit and a PTFE rod, which was drilled through with a stainless-steel capillary.

Detection system

Fig. 2a shows the extraction detection system as described earlier[4,6]. A peristaltic pump (Pharmacia, Uppsala, Sweden) was used to deliver the DAS-containing solution and the organic extraction solvent and also to control the flow passing through the detector cell. Reaction and extraction took place in a 14-turn glass coil (1 mm I.D.). Phase separation was carried out with a conventional Technicon phase separator provided with PTFE tubing of 1.5 mm I.D. with a hole cut into it to permit the aqueous phase to escape. From the hole to the detector cell, PTFE tubing of

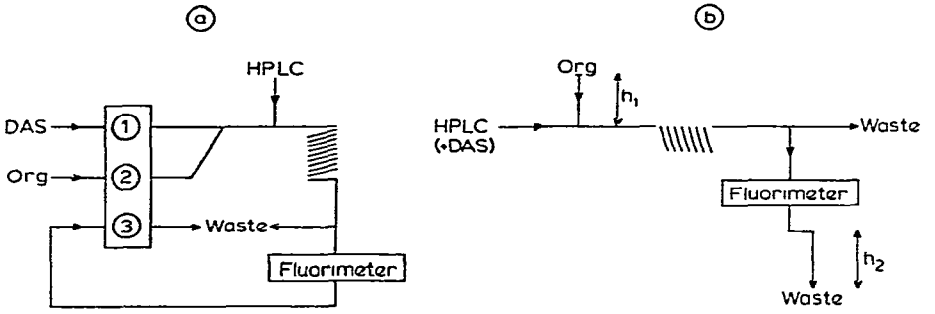

Fig. 2. Post-column ion-pair extraction systems. (a) Flow-rates (ml/min): 10^{-4} *M* DAS, 0.6; organic solvent, 0.6; through the flow cell, 0.3; HPLC, 1.0. (b) Flow-rates (ml/min): organic solvent, 0.2; through the flow cell, 0.14; HPLC, 1.0. $h_1 = 60$ cm; $h_2 = 80$ cm.

0.3 mm I.D. was inserted into the first tubing. In order to reduce mixing in the separator, the removal of the aqueous phase was accelerated by inserted a glass tube of 0.8 mm I.D. into the waste pipe just above the hole in the PTFE tubing. This phase separator is similar to that described by Lawrence *et al.*[5]. A Perkin-Elmer Model 3000 fluorescence spectrophotometer was used for detection of the fluorescent ion pairs ($\lambda_{exc.} = 383$ nm; $\lambda_{em.} = 452$ nm).

Fig. 2b shows schematically the modified extraction detector. Here, DAS was added to the mobile phase prior to the column and the organic extractant was added to the column effluent by means of gravity, using a separating funnel placed at a suitable height above the extraction detector. Part of the organic phase was drawn through the detector cell by means of the siphon principle.

Analysis of plasma and serum

After thawing of blank plasma or serum, an aliquot was spiked with an aqueous standard solution of secoverine in a ratio of plasma or serum to water of 9:1. Volumes of 200–1000 μl of this mixture were injected on to the pre-column with water as the mobile phase and with the switching valve in the waste position. Volumes larger than 200 μl were introduced as intermittent 200-μl injections at intervals of about 1 min.

About 5 min after the last injection, the valve between the two columns was switched on-line and secoverine was eluted with dioxane–0.1 *M* sodium dihydrogen phosphate solution, adjusted to pH 3.2 with 85% phosphoric acid, (10:90, v/v) containing $1.5 \cdot 10^{-5}$ *M* DAS; the flow-rate was 1.5 ml/min.

RESULTS AND DISCUSSION

Extraction system

For part of the optimization experiments, the system[4] depicted in Fig. 2a was used successfully. However, it was observed that the pulsating peristaltic pump made a considerable contribution to noise. The system shown in Fig. 2b was therefore developed as an alternative. In agreement with the data for a number of other amines[6], it was found that the addition of DAS to the mobile phase prior to the column caused only a small increase in the retention of secoverine. For example, with methanol–0.1 *M* phosphate buffer pH 3.2 (40:60, v/v) as the mobile phase, the capacity factor

of secoverine increased from 1.8 to 2.3. The use of gravity (or a syringe pump) for the addition of the organic solvent to the column effluent reduced the detector noise about 4-fold in comparison with the noise observed with the pulsating peristaltic pump.

Table I compares the performance of a number of chlorine-containing extraction solvents. The widely different values of the signal-to-noise ratios were shown to be caused by differences in peak heights, not by different noise levels. The best results were obtained with 1,2-dichloroethane and dichloromethane. It is known that "acidic" solvents of this type easily form hydrogen bonds, which is advantageous for the extraction of ion pairs. Dichloromethane often gave rise to bubble formation in the detector owing to its high volatility; therefore, 1,2-dichloroethane was the preferred solvent. Because the background signal and, consequently, part of the noise are due to co-extraction of DAS, the effect of some relatively apolar solvents on the signal-to-noise ratio was investigated. Unfortunately, the addition of 5% of *n*-hexane or isooctane to 1,2-dichloroethane decreased the peak height, but the noise level remained the same.

TABLE I

EFFECT OF THE NATURE OF THE ORGANIC SOLVENT ON THE RELATIVE SIGNAL-TO-NOISE RATIO OF THE DAS–SECOVERINE ION PAIR

Detection system: see Fig. 2a.

Solvent	*Relative signal-to-noise ratio*
Dichloromethane	115
Trichloromethane	42
Tetrachloromethane	0
1,2-Dichloroethane	125
1,1,2-Trichloroethane	85
1,1,2,2-Tetrachloroethane	70
1,2-Dichloropropane	18

Increasing the flow-rate of 1,2-dichloroethane lowered the signal-to-noise ratio. The optimum at relatively low flow-rates, *i.e.*, 0.2–0.4 ml/min at a flow-rate of the mobile phase of 1 ml/min, can be explained by a higher ion-pair concentration in the organic phase. In contrast to the findings of Lawrence *et al.*[5] for the DAS–hydroxyatrazine ion pair, a low ratio of organic to aqueous phase is advantageous for the DAS–secoverine ion pair because of its relatively high distribution coefficient (>50). The fraction of the organic phase passing through the detector cell also influences the peak height. Doubling this fraction from 35 to 70% gave a 40% increase in the peak height. (A still higher fraction readily gives rise to phase-separation problems, with the consequence that part of the aqueous phase is also drawn through the detector cell.) This phenomenon, which cannot easily be explained as the detector is concentration sensitive, has also been observed by Karlberg and Thelander[9] and Scholten *et al.*[14].

Chromatographic system

The relatively apolar tertiary amine secoverine is efficiently extracted as its DAS ion pair. However, the fact that an aqueous mobile phase with a relatively large

content of a modifier is required for the elution of this ion pair in a reversed-phase system is a drawback. This has a pronounced effect of the extraction of the counter ion. In order to decrease the modifier content of the mobile phase, a polar bonded phase was chosen as the stationary phase. Table II gives data on the retention of secoverine on CN-bonded silica, and on the signal-to-noise ratio and noise level for some mobile phases. In contrast to the findings reported in Table I, in this instance mutual differences in noise level are seen to contribute significantly to the differences observed in the signal-to-noise ratio. For the rest, dioxane is seen to be more suitable than methanol as a modifier because the former gives a higher signal-to-noise ratio at similar retention times. It should be noted that a further decrease in the modifier content can be achieved by using more polar phases such as OH- or NH_2-bonded silica as the stationary phase. However, preliminary experiments carried out with columns packed with these stationary phases showed them to be less efficient than the CN-bonded silica columns.

TABLE II

EFFECT OF THE NATURE AND CONTENT OF THE MODIFIER IN THE MOBILE PHASE ON THE RETENTION TIME OF SECOVERINE AND ON THE RELATIVE SIGNAL-TO-NOISE RATIO OF THE DAS–SECOVERINE ION PAIR

Chromatographic conditions: CN-bonded silica column; modifier–0.1 *M* phosphate buffer (pH 3.2) as mobile phase; flow-rate 1 ml/min. Detection system: see Fig. 2a.

Modifier	*Retention time (min)*	*Relative signal-to-noise ratio**	*Relative noise*
None	>45	410	2
5% dioxane	26	257	3
10% dioxane	14	170	5
15% dioxane	7.5	125	15
20% methanol	>45	185	10
40% methanol	7	85	20

* Determined by means of plug injection.

The DAS concentration of the mobile phase was varied over the range 10^{-4}–$1.5 \cdot 10^{-5}$ *M*. For an amount of 20 ng of secoverine injected, the peak heights were found to decrease below about $5 \cdot 10^{-5}$ *M* DAS, whereas the signal-to-noise ratio showed a small increase. A $1.5 \cdot 10^{-5}$ *M* DAS solution was therefore used routinely.

Under optimized conditions, band broadening due to the extraction detection system was found to be $\sigma_t = 7$ sec (at a flow-rate of 1.5 ml/min). The repeatability of the present system in the nanogram range is about 2% (relative standard deviation) ($n = 6$). The detection limit for secoverine in aqueous standard solutions at a signal-to-noise ratio of 2:1 is *ca.* 20 pg and the linear range is at least 4 decades.

Analysis of plasma and serum

Insertion of a pre-column in a HPLC system is very useful for the clean-up of, and pre-concentration from, biological samples. In this way, the pre-treatment of such samples, which is often time consuming, can be simplified or even omitted. In the present investigation, an easily replaceable pre-column containing a short (1–2 mm) plug of stationary-phase material, constructed according to Van Vliet *et al.*[13] was chosen. CN-, C_8- and C_{18}-bonded phases were all found to be suitable packing

materials for this pre-column, as far as clean-up and trace enrichment of secoverine from blood samples are concerned, as the drug was not eluted from any of the three materials tested even with 15 ml of water. However, when using the previously selected mobile phase containing a small proportion of organic modifier (10% dioxane; see above), elution of the DAS–secoverine ion pairs from the pre-column filled with C_8- and C_{18}-bonded phases was too slow and, consequently, band broadening was intolerably high. On the other hand, as is to be expected, no such problems were encountered with the CN-bonded silica. To quote a typical result, with the CN-, C_8- and C_{18}-bonded phases as packing materials for the pre-column, band broadening due to the total analytical system was σ_t = 15, 33 and 63 sec, respectively. Under the pertinent conditions, σ_t was also 15 sec for the system without a pre-column.

Fig. 3 shows that the recovery of secoverine after pre-concentration from even 1 ml of serum was 100%. The rise of the baseline in Fig. 3b and c must be ascribed to the gradient caused by the change in mobile phase composition from water to the dioxane–phosphate buffer (10:90) mixture. For the determination of low secoverine concentrations, it is recommended that the dioxane content of the mobile phase is lowered slightly, thereby increasing the retention of the DAS–secoverine ion pair. The performance of the system can be further improved by dissolving DAS in the water used during the trace-enrichment step, which causes the "dip" in the chromatogram just prior to the secoverine peak to disappear. The chromatograms in Fig. 3 clearly illustrate the high selectivity obtained by the combined use of pre-column technology and the extraction–detection principle. This is also apparent from the fact that the chromatograms obtained after pre-concentration from serum samples are nearly identical with those recorded after pre-concentration of secoverine from aqueous standard solutions.

CONCLUSION

The extraction detector described in this paper is very simple and reliable. The noise of the present system, which self-evidently can also be used in flow-injection analysis, is significantly lower than that of the system with a pulsating peristaltic pump. It can also be concluded that a very sensitive and selective determination of the prospective drug secoverine can be achieved by ion pairing of this tertiary amine with DAS and subsequent separation of the ion pair from excess of reagent by means of continuous extraction.

The combination of the extraction detector with an HPLC system consisting of a suitable pre-column and an analytical column can be used for the direct analysis of secoverine in biological samples. The method currently used in our laboratory for the determination of secoverine in plasma is gas chromatography–mass fragmentography. This relatively expensive method demands a laborious pre-treatment and has a detection limit of 40 pg/ml in plasma. Current research is directed at making the present technique useful as a routine method with about the same sensitivity. The main problem still to be solved is how to prevent an undue increase in the back-pressure of the pre-column and/or clogging of this column during trace enrichment of a series of samples. Promising procedures include filtration of the plasma and serum samples over a suitable disposable filter and back-flushing of the pre-column after each pre-concentration step.

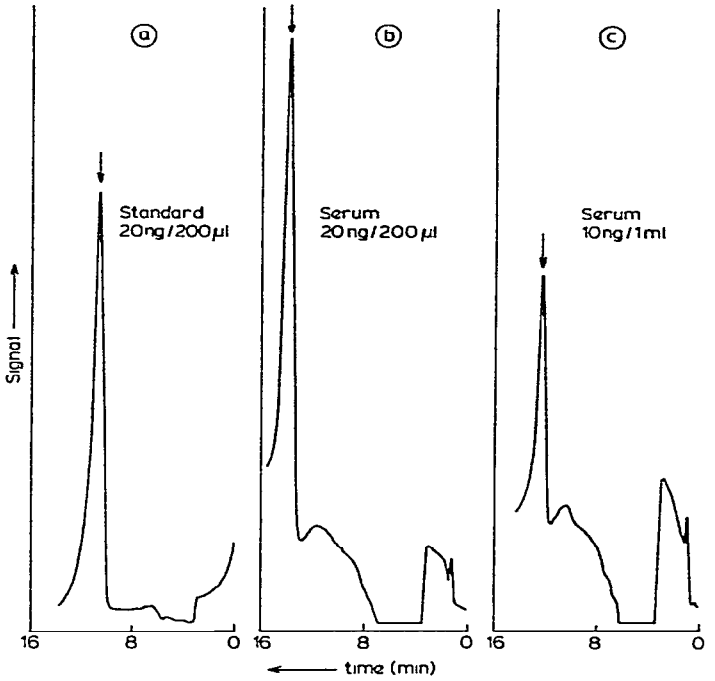

Fig. 3. Chromatograms of secoverine-containing samples. (a) Aqueous standard solution of 100 ng/ml of secoverine; direct injection of 200 µl. (b) Spiked serum sample containing 100 ng/ml of secoverine; pre-concentration from 200 µl. (c) Spiked serum sample containing 10 ng/ml of secoverine; pre-concentration from 1 ml. Note that the amount of secoverine injected in chromatogram (c) is half of that in chromatogram (b).

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