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BIOLOGICAL DETECTORS IN LIQUID CHROMATOGRAPHY

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SUMMARY

The applicability of isolated organ preparations as chromatographic detectors was demonstrated. An appropriately designed detector cell (biological detector) applicable in both on-line and off-line modes was developed. Deproteinized sera from healthy volunteers and schizophrenic patients and human amniotic fluid were fractionated by gel-permeation, ion-exchange and reversed-phase liquid chromatography. Ultraviolet absorption and biological activity were compared. The results show that isolated organ preparations selected according to the needs of particular experiments meet the essential criteria of conventional chromatographic detectors. The use of isolated organs allows the detection of biologically active substances in a matrix of physico-chemically closely related, but biologically distinct, fluid components Biological detectors may also provide valuable additional information concerning the chemical structure of biologically active agents in an early stage of isolation.

INTRODUCTION

The selection of the appropriate detector for a particular chromatographic analysis is a step of paramount importance, and often it is a difficult task. Often the substance to be separated is present in a complex mixture of compounds whose physico-chemical properties are closely related to those of the substance to be detected. Although today a considerable number of highly selective detectors are available, primarily owing to their high specificity none is suitable as a general-purpose instrument [1–3]. The chromatographer therefore has thus to find the most appropriate detection system in every instance.

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Thus, e.g., one usually encounters substantial difficulties when a compound with given biological activity has to be separated from, and detected in, one of the body fluids, which usually contains a complex mixture of compounds with closely related characteristics.

Detection of biological activity in the effluent or in collected fractions is well known in chromatography [4–13]. For example, in order to detect the presence of sex attractants of different animals in gas chromatographic effluents, the carrier gas was driven through chambers containing the appropriate animals (American cockroach, silk spider, gypsy moth, etc.) [14–17]. Characteristic movements of the animal (wing movement, whirling dance, etc.) served as indicators in those experiments. These detection methods are highly sensitive, e.g., 20 ng of sex attractants of the American cockroach were detected [14]. However, in those methods the 'detector response' was not an electrical signal which could be recorded, but the visual observation made by the chromatographer. This response could hardly be expressed numerically and cannot offer information about the chemical structure of the substance detected.

In this paper, we intend to show that appropriately selected isolated organ preparations, used extensively in several fields of biomedical research, can conveniently be used as chromatographic detectors (CDs) under suitable conditions. An up-to-date, sensitive CD is expected to meet the following criteria [1–3]: it must be sensitive enough (e.g., the sensitivity of a modern UV detector is about 10–20 ng/ml [3]); it must be highly selective; it must respond quickly to changes in the composition of the effluent; it must generate a signal that is proportional to the amount or concentration of the sample to be detected; and noise and drift must be negligible.

As it will be illustrated below, using isolated organ preparations we can build a detection device (called a biological detector) which shares many of these criteria of up-to-date and sensitive CDs. In addition, by using isolated organs as CDs, one may obtain valuable additional information concerning the chemical structure [18–26] and possible physiological role [27–33] of the detected substance. For example, vasa deferentia are richly supplied with highly sensitive opiate receptors in mice [34–36]. This is indicated by the capability of the isolated mouse vas deferens (MVD) preparation to detect met-enkephalin, a widely known endogenous opiate compound, in a concentration as low as 35 nmol/l or 20 ng/ml. On the other hand, research conducted on endogenous opiate compounds soon revealed that manifestation of the opiate activity of a molecule requires the simultaneous presence of a group positively charged at physiological pH values, an aromatic ring coplanar with the positively charged group and a peptide chain of appropriate length between the aromatic ring and the positively charged group [36–41]. As a consequence, if a compound is found to display opiate activity, we may be sure that it possesses the structural characteristics mentioned above. As various isolated organs respond differently to chemical substances with distinct structural characteristics, further informa-

tion on the molecular structure of a certain compound can easily be collected by comparing its biological activity on different isolated organ systems. For example, by the comparative use of MVD and guinea-pig ileum assay systems, information on the flexibility or rigidity of the detected compound can be obtained [36]. The effect of an opiate-active compound on MVD is demonstrated in Fig. 1, where it is also demonstrated that the opiate effect can be antagonized by naloxone, an opiate antagonist.

Apart from MVD, isolated rat uterus strip (RUS) preparations were the second type of isolated organs that we used as a CD. This preparation has also the capability of responding with remarkable sensitivity to compounds with appropriate chemical structure (Table I). The effects that can be elicited on a RUS preparation by chemical stimuli are demonstrated in Fig. 2. As can be seen, different biologically active agents may affect the organ preparations differently: they may increase or decrease the tone, amplitude or the frequency

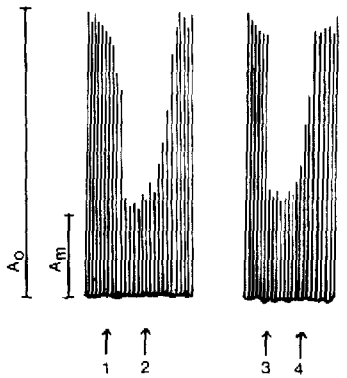

Fig. 1. Left: inhibiting effect of met-enkephalin on contractions of isolated mouse vas deferens. Right: antagonizing effect of morphine antagonist naloxon on the inhibiting effect of met-enkephalin. A_c, Amplitude of contractions; A_m, amplitude of contractions of active compound [inhibition (%) $= 100 - (A_m/A_0) \cdot 100$]. 1 and 3, addition of met-enkephalin, 2, wash-out; 4, addition of naloxon.

TABLE I

ACTIVITY OF SOME COMPOUNDS ON THE ISOLATED RAT UTERUS STRIP

ID_{50}: active compound causes inhibition of 50% at this concentration.

Compound	ID_{50} (mol/l)	Effect
Oxytocin	$2 \cdot 10^{-10}$	Contraction
Histamine	10^{-7}	Relaxation
Epinephrine	10^{-7}	Relaxation
Norepinephrine	10^{-7}	Relaxation
Acetylcholine	10^{-7}	Contraction

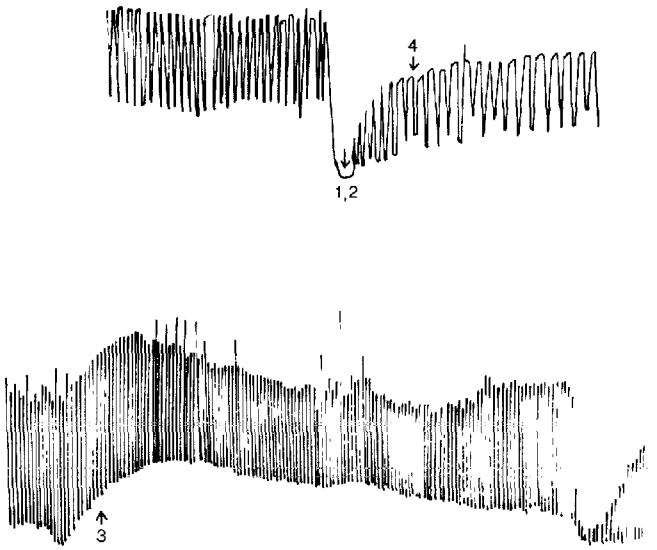

Fig. 2 Different effects of biologically active components on rat uterus strip. 1, Decrease in tone; 2, decrease in amplitude; 3, increase in tone; 4, change in frequency.

of the contractions, etc. Application of a biological detector in the analysis of sera of schizophrenic patients and in the isolation of biologically active fractions obtained from an acidic acetone extract of sow ovaries were reported in previous papers [42–44]. In those experiments we demonstrated that the sole use of a conventional (UV) detector would not allow the presence of biologically active compounds to be recognized in the large matrix of compounds whose chemical and physico-chemical properties were closely related to or even identical with those of the biologically active compounds.

In this paper we report on features of the biological detector and give various examples of its practical use.

EXPERIMENTAL

Features of the biological detector

The principal component of biodetection systems is the flow-through-type detector cell which can be operated in both off-line and on-line modes. The detector cell shown in Fig. 3 is a flow-through-type thermostated glass vessel of 1-ml volume. The electrodes of an electrostimulator are fixed at the top and the bottom of the cell. Continuous or periodic flow of the washing solution through the detector cell is maintained by a peristaltic pump. A continuous flow is especially important when applying organ preparations with an intensive metabolism (e.g., uterus strip). In order to maintain a good functional state of these organs it might be necessary to wash the detector cell continu-

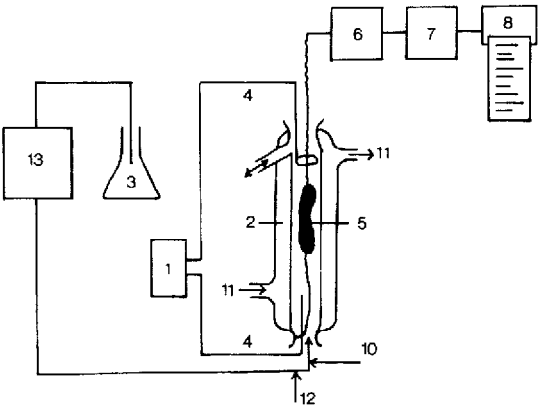

Fig. 3. Flow-through-type detector cell allowing off-line operation of a biodetector 1, Electrostimulator (Medicor); 2, detector cell; 3, washing solution; 4, electrodes; 5, isolated organ; 6, extensometer (Rolitron); 7, bioforcemeter (amplifier, Rolitron); 8, recorder (OH-814, Radelkis); 10, carbogen gas [CO-O_2 (5:95)] inlet; 11, thermostat; 12, site of sample injection; 13, peristaltic pump.

ously with an appropriate physiological solution. Without such measures, isolated organs with an intensive metabolism would rapidly deteriorate, thereby making impossible their use as biological detectors. Periodic flow of the physiological washing solution is required when the active component to be detected does not evolve its activity immediately, but during a longer period (e.g., prolactin can evolve its activity on RUS only 20–30 min after injection [45]). Biological responses (e.g., contractions) of isolated organs are registered by an appropriate recording instrument [e.g., a potentiometric recorder (Radelkis) connected to an extensometer (Rolitron)].

If discontinuous registration of biological activity elicited by components in the separately collected fractions is required, aliquots of each fraction should be injected one-by-one into the detector. In this instance the system operates in an off-line manner. As the organ preparation may be flushed continuously with a physiological washing solution during the whole experiment in this system, this experimental set-up allows the application of isolated organs of intensive metabolism as biological detectors.

The system shown in Fig. 4 represents a variety which, apart from securing continuous flushing of the detector cell with a physiological solution, may also be connected directly to the chromatographic column, as an on-line detector. If the flow-rate of the eluent is high enough to prevent the accumulation of toxic metabolic products in the surroundings of the biodetector and the composition of the effluent is an appropriate one (identical with or near to the composition of the feeding solution of the isolated organ), the auxiliary pump (13 in Fig. 4) does not need to operate. In this instance, the composition of the effluent entering the biodetector remains unchanged.

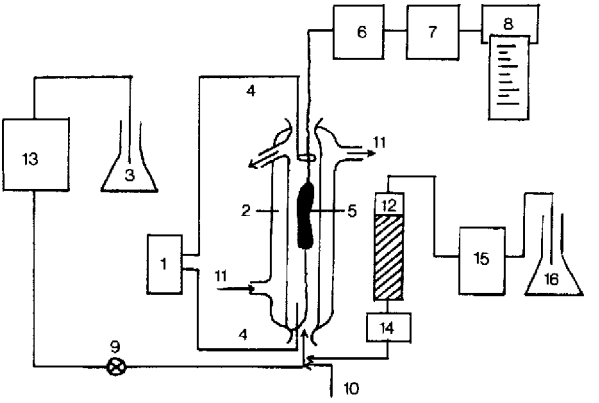

Fig. 4. Flow-through-type detector allowing on-line operation of biodetectors. 1, Electrostimulator; 2, detector cell; 3, auxiliary solution; 4, electrodes; 5, isolated organ; 6, extensometer; 7, bioforcemeter; 8, recorder; 9, valve; 10, carbogen gas inlet [CO_2–O_2 (5:95)]; 11, thermostat inlet and outlet; 12, chromatographic column; 13, peristaltic pump, 14, UV detector; 15, peristaltic pump; 16, reservoir for the eluent.

If the flow-rate of the effluent is lower than is desirable, an auxiliary solution should be added to the effluent (the composition of which should be identical with that of the chromatographic eluent) in a amount that increases the flow-rate of the effluent to the value desired. In order to accomplish this, the system has to be mounted with an auxiliary pump. The same pump can be used to add auxiliary solution (of appropriate composition) to the effluent if the concentrations of compounds needed to maintain the state of the isolated organ are lower than desired.

If the composition of the chromatographic eluent deviates from that of the feeding solution of the isolated organ to an extent that endangers proper functioning of the biodetector (the isolated organ), the effluent has to be changed post-column to a solution tolerable for the isolated organ by the addition of an auxiliary solution of appropriate composition to the effluent. Occasionally, both the amount and quality of information obtained may be significantly enhanced by placing a conventional detector (e.g., a UV detector) between the chromatographic column and the biological detector (Fig. 4). The simultaneous determination of multiple biological activities initiated by various effluent components can also be accomplished by using an on-line system mounted with an appropriately constructed distribution head. Several isolated organ preparations used simultaneously in parallel connection, and displaying a biological response elicitable by specific signals represented by various substances in the effluent, are required for this purpose.

Applications of biological detectors

To check the linearity, sensitivity and reproducibility of the biological detector activities [inhibition effect: $I\ (\%) = 100 - (A_m/A_0) \cdot 100$, where A_0 and

A_m are amplitudes of contractions before and after injection of the compounds being investigated, respectively] evolved by met-enkephalin (Sigma) on isolated MVD were measured. The results are shown in Table II and in Fig. 5. The preparation of the smooth muscle strip, composition of Krebs–Ringer solutions and parameters of electric stimulation are specified elsewhere [34,43].

Deproteinized sera from healthy volunteers (controls) and from schizophrenic patients were separated on a Sephadex G-25 column [42,44]. The biological activity and UV light-absorbing capability (at 245 nm) of the separated fractions were detected with an MVD system (with off-line detector; the volume of samples injected into the detector cell was 150 μl) and a Uvicord S detector (254 nm) (LKB), respectively. The preparation of the smooth muscle

TABLE II

DOSE-RESPONSE DATA OBTAINED ON MOUSE VAS DEFERENS BY MET-ENKEPHALIN

Concentration of met-enkephalin (nmol/l)	Inhibition (mean ± S.D.) (%)	Concentration of met-enkephalin (nmol/l)	Inhibition (mean ± S.D.) (%)
20	2.4 ± 0.15	310	62.9 ± 1.82
30	5.3 ± 0 29	550	82.9 ± 2.57
40	8.2 ± 0.36	750	88.7 ± 2.31
50	11.4 ± 0 49	1000	97.1 ± 2.80
80	24.3 ± 0.97	1100	98.0 ± 3.00
180	51.5 ± 1 80	1200	98.9 ± 2.90

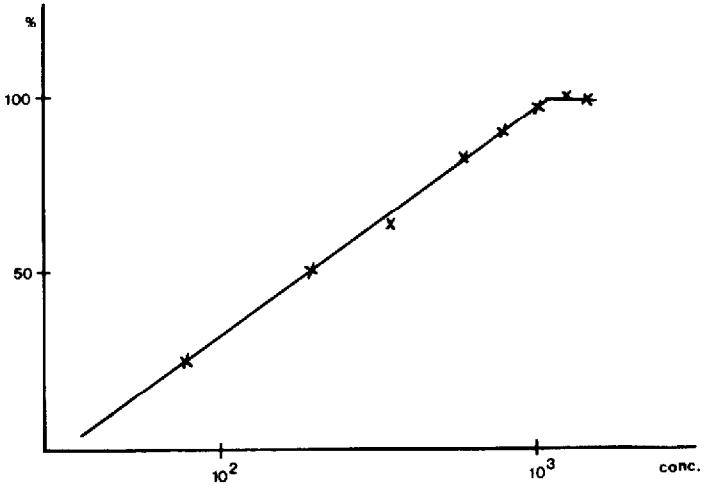

Fig. 5. Dose–response curve obtained on isolated mouse vas deferens by met-enkephalin. Abscissa, concentration of met-enkephalin (nmol/l), log scale; ordinate, inhibiting effect caused by met-enkephalin (number of experiments, 30).

strip and composition of Krebs–Ringer solution were described elsewhere [34,43]. The chromatographic parameters were as follows: column, 50 cm × 0.8 cm I.D.; eluent, 0.9% sodium chloride; flow-rate, 0.6 ml/min; volume of collected fractions, 1.0 ml; chart paper speed, 40 mm/h; sample volume, 3.0 ml; and biological detector, isolated MVD preparation. In another series of experiments, amniotic fluid obtained during the 16th–22nd weeks of pregnancy by amniocenthesis was centrifuged (4 °C, 7800 *g*, 20 min). The supernatant was separated by several chromatographic techniques.

Gel-permeation chromatography on Sephadex G-25 (fine) gel. The chromatographic parameters were as follows: column, 75 cm × 1.3 cm I.D.; eluent, Krebs–Ringer solution of composition described previously [42]; flow-rate, 2.0 ml/min; detection, 254 nm (Uvicord S); and sample volume, 20 ml of amniotic fluid. The biological activity of the effluent was determined on isolated RUS (its preparation was described in ref. 42, but in the experiments presented here RUS preparations contracting spontaneously were applied) with the biological detector operating in an on-line manner.

Ion-exchange chromatography. The fraction inhibiting contractions of RUS preparation was eluted from a Sephadex G-25 column with K_{av} values of 0.6–0.85 [$K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume and V_0 and V_t are the dead volume and total volume of the column, respectively]. This fraction was further separated on a Whatman CM32 column. The chromatographic parameters were as follows: column, 40 cm × 1.3 cm I.D.; eluent 1, 0.2 *M* sodium chloride, pH 4.0 (180 ml); eluent 2, 2.0 *M* sodium chloride, pH 4.0 (300 ml); flow-rate, 3.0 ml/min; detection, Uvicord S (254 nm); sample volume, 50 ml; and fraction volume, 20 ml. Fractions were desalted with Dowex 50-X8 gel, lyophilized and dissolved in Krebs–Ringer solution (10 ml) and their biological activity on isolated RUS preparation was determined with a biological detector operating in an off-line manner (200-μl samples were injected into the detector cell). The active fraction was concentrated to half its volume on an Amicon UM 05 membrane.

Reversed-phase liquid chromatography (LC). The biologically active fraction obtained as described in the previous section was purified on a Whatman Magnum 9 ODS column (500 mm × 4 mm I.D.). The chromatographic parameters were as follows: eluent, acetonitrile–*n*-propanol–water (4:20:76, v/v); flow-rate, 5.0 ml/min; pump, Liquopump 312 (Labor-MIM); detector, OE-309 UV (Labor-MIM) (254 nm); and sample volume, 5.0 ml. The fractions obtained were lyophilized, dissolved in Krebs–Ringer solution and their biological activity on isolated RUS preparation was determined with a biological detector operating in an off-line manner (300-μl samples were injected into the detector cell).

RESULTS

On checking the sensitivity, reproducibility and linearity of the biological detector, we found the biological detector to be highly sensitive (e.g., met-

enkephalin applied in this experiment can be detected at concentrations as low as 30 nmol/l), and the detector response is a linear function of the logarithm of the concentration in the range 40–1000 nmol/l (Fig. 5); the reproducibility is shown in Table II.

As can be seen in Fig. 6, samples from both normal and schizophrenic patients could be resolved by SG-25 chromatography into three distinct peaks with almost identical K_{av} values. In the schizophrenic profile, however, an additional peak with $K_{av}=0.51$ could also be observed, which was regularly absent from the normal samples. The ratio of the corresponding peaks from the two samples also differed to some extent. In general, however, no substantial difference between the two profiles could be observed by conventional detection at 254 nm alone. In contrast, the corresponding fractions from the two samples showed striking differences when their actions on MVD were compared. The control fractions were completely inactive on MVD, whereas the schizophrenic fractions markedly inhibited contractions of MVD. This experiment clearly demonstrates the occasional superiority of a biological over a conventional detector. The sole use of the conventional detector would not allow the significant differences in the composition of the two samples to be recognized. As the components having MVD activity in schizophrenic sera were present in a large matrix of compounds whose chemical and physicochemical properties were closely related or even identical, the very small amount of the MVD-active component(s) in schizophrenic sera would have remained undetected by using a conventional detector only. From the results presented above, the highly specific character of the biological detector used can also be recognized. At this stage of purification, none of the schizophrenic fractions were homogeneous in composition. However, the use of a biological detector allowed the detection of the biologically active agents, even in these highly heterogeneous fractions.

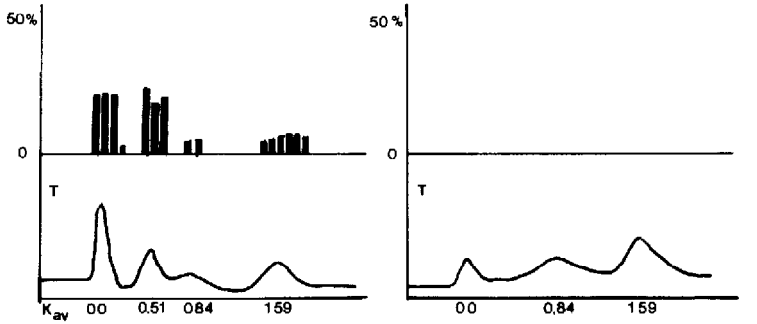

Fig 6. Sephadex G-25 profile (bottom) and biological activity on isolated mouse vas deferens (top) of schizophrenic (left) and non-schizophrenic (right) sera. Abscissa, K values of the fractions; ordinate, inhibiting effect of the fractions (top) and transmittance at 254 nm (bottom) For chromatographic parameters, see Experimental.

Fig. 7 shows the gel-permeation chromatographic pattern of the amniotic fluid and biological activity of the effluent. The chromatogram shows the absorbance at 254 nm, and the upper part presents the pattern produced by the biological detector. The fraction eluting with $K_{av}=0.6$–0.7 inhibits contractions and enhanced tone. Fraction eluting with $K_{av}=0.70$–0.85 completely inhibits the contractions of RUS and decreases the tone.

Fig. 8 shows the ion-exchange chromatogram of the biologically active Sephadex fraction. The biological activity of the fractions can be seen. The first fraction (eluting with 0.2 *M* sodium chloride) depresses the contractions of the RUS preparation and evokes a complete but reversible inhibiting and tone-enhancing effect. The biological activity of the second peak (eluting with 2.0 *M* sodium chloride) will be discussed in another paper; the third peak proved to be completely inactive towards the RUS preparation.

The results of the reversed-phase LC separation of the biologically active ion-exchange fraction (0.2 *M* sodium chloride) obtained with UV and biological detection are summarized in Fig. 9. The peak eluting at 32 min inhibits the contractions of both the RUS preparation and human uterus preparation while the peak eluting at 53 min elevates tone.

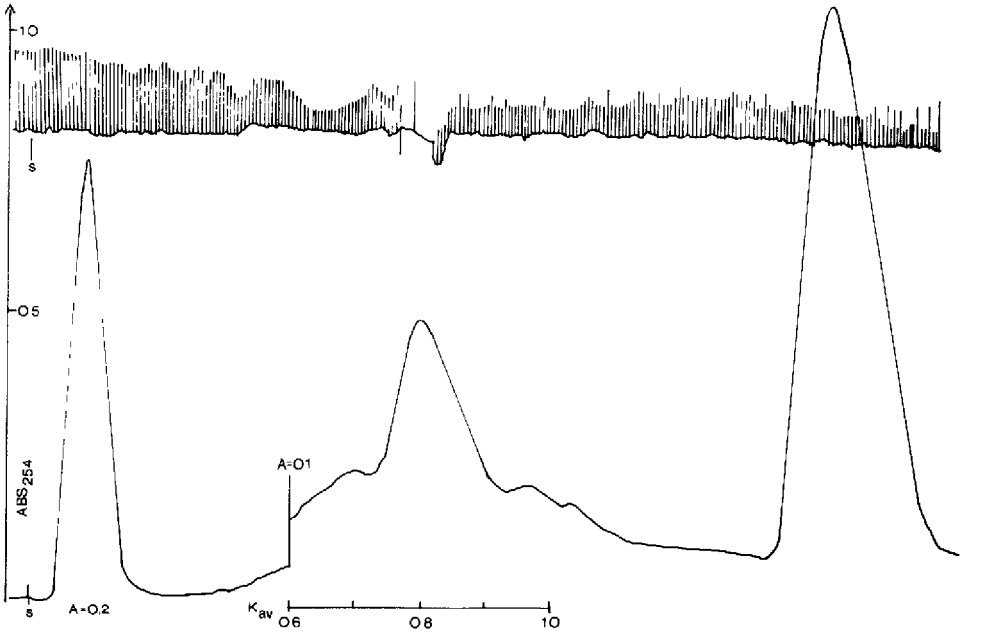

Fig. 7. Gel chromatographic pattern of human amniotic fluid on Sephadex G-25 gel and biological activity of the chromatographic effluent on isolated RUS preparation. The lower part shows the chromatogram obtained with UV detection (abscissa, *K* values; ordinate, absorbance at 254 nm); the upper part shows the biological activity determined with the biological detector working in an on-line manner. For chromatographic conditions, see Experimental.

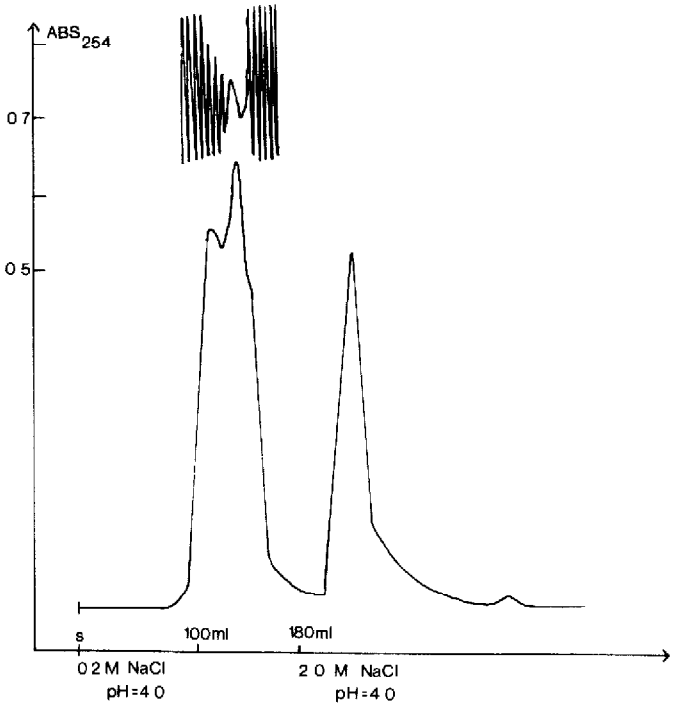

Fig. 8. Ion-exchange chromatogram of the biologically active gel chromatographic fraction performed on Whatman CM-32 gel. The lower part shows the chromatogram obtained with UV detection (abscissa, elution volume in ml; ordinate, absorption at 254 nm); the upper part shows the effect of biologically active fraction on RUS preparation. For chromatographic conditions, see Experimental.

With subsequent use of gel-permeation, ion-exchange and reversed-phase LC, a fraction from human amniotic fluid, which reversibly inhibits the activity of both human and rat uterus, was partially purified [46–48]. By the simultaneous application of conventional and biological detection and comparing the data obtained by these two methods, useful information can be obtained even in early stage of separation work, whether the compound to be separated is identical with or distinct from compounds of already known structure and activity. This information can help in making a decision whether it is worth continuing the separation or not (whether there is a compound of unknown structure present in the partially purified fraction or not).

Comparison of the chromatographic elution data and the biological activity of this fraction and those of substances of known structure and biological activity (which are able to affect the functioning of the uterus smooth muscle) shows that this partially purified fraction is not identical with relaxin, vasoactive intestinal peptide, prolactin, epinephrine, prostaglandins F or E [47].

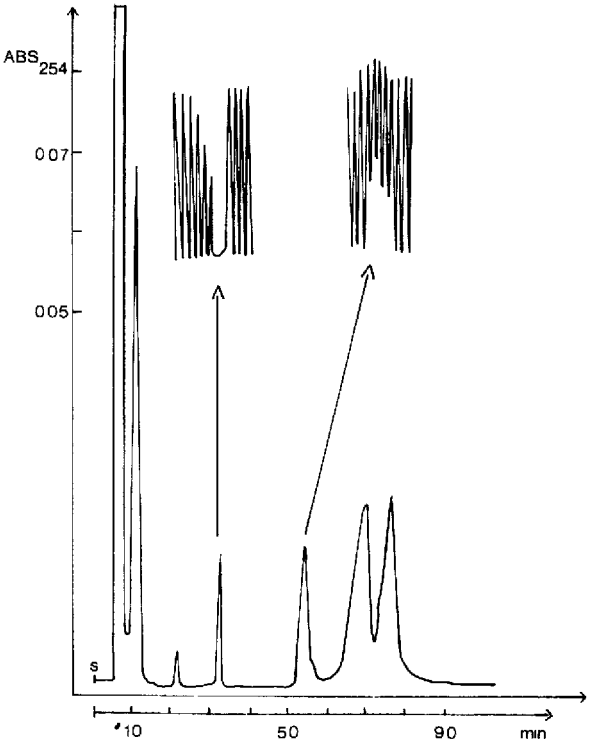

Fig. 9. Separation of biologically active ion-exchange fraction on a reversed-phase LC column (Whatman Magnum 9). The lower part shows the chromatogram obtained by UV detection (abscissa, elution time in min; ordinate, absorption at 254 nm), the upper part shows the effects of biologically active LC fractions on RUS preparation. For chromatographic parameters, see Experimental.

This subsequent use of different chromatographic techniques in the purification of amniotic fluid enlightens limit of application of biological detectors. Chromatographic effluents containing components which adversely affect the proper functioning of the isolated organ (salts in high concentration, organic solvents, etc.) must not enter directly into the biological detector. Such components must be eliminated (ultrafiltration, dialysis, vacuum evaporation, lyophilization, etc) before biological detection. In this instance biological detectors can only be applied in an off-line manner.

DISCUSSION

The results of this and previous work [42,46,47] demonstrate that isolated organ preparations may occasionally be applied as CDs with excellent success, because isolated organs share essential properties with up-to-date conven-

tional CDs. (a) They are highly sensitive; e.g., met-enkephalin can be detected by MVD at a concentration of 35 nmol/l or 20 ng/ml [42,43], in contrast to 10–20 ng/ml, the desired sensitivity of a modern UV detector [3]. Taking into account the volume of the detector cell applied in these experiments, the amount of met-enkephalin that we could detect with the aid of MVD was as low as 0.03 nmol. This sensitivity is comparable to that of a UV detector (0.07 nmol) applied in an LC system which was used for met-enkephalin separation as described recently [49]. (b) They respond almost as quickly as conventional detectors to changes in the effluent composition. (c) Their response is proportional to the logarithm of the concentration of the substance to be detected. (d) They are highly specific, by being able to detect a biologically active agent in an occasionally highly complex matrix of compounds with similar physicochemical and chemical properties, but dissimilar biological activity.

Biological detectors can be operated in off-line manner when the biological activity of individual fractions obtained by a conventional separation procedure is to be determined discontinuously by injecting them one-by-one into the detector cell. The use of a flow-through-type cell allows not only organ preparations with intensive metabolism to be applied as biodetectors, but also biological detectors to be connected directly to the chromatographic column and operated in an on-line manner. On the other hand, completing the system with a properly constructed distribution head allows the simultaneous registration of multiple biological activities with the aid of several biological detectors connected in parallel. A conventional detector fitted between the chromatographic column and the biological detector may increase the amount of information obtained. On-line biological detection can also be accomplished by carrying out auxiliary measures even when the composition of the eluent is aphysiological.

The application of biological detectors seems to be especially promising in the following fields: (a) in extensive areas of physiological, pharmacological and biological research; (b) occasionally in revealing the molecular etiology of various pathological conditions; (c) in all instances, when a compound with optional biological activity has to be detected in a complex matrix of compounds in which we are not interested; (d) in cases when quickly obtainable preliminary characteristics of a compound are required before its complete isolation.

Naturally, like any other detectors, biological detectors also have shortcomings and limits of application. For example, isolated organs are susceptible to external influences and their application needs strict safeguarding of physiological conditions. The presence of toxic agents or organic solvents in their environment, even in trace amounts, may easily kill the isolated organ preparations. Hence such contaminants should be eliminated, or at least neutralized prior to biodetection. This may not always be easy, and may require excess

work. However, the several advantages offered by biological detection usually deserve the investment of extra efforts.

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